FUNDAMENTAL PRINCIPLES
of
BACTERIOLOGY

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Fundamental Principles
of
Bacteriology

By

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Second Edition
Sixth Impression

McGRAW-HILL BOOK COMPANY, Inc.
NEW YORK AND LONDON
1943

FUNDAMENTAL PRINCIPLES OF BACTERIOLOGY

PRINTED IN THE UNITED STATES OF AMERICA

THE MAPLE PRESS COMPANY, YORK, PA.

To

CECELIA DAVERSO SALLE

this book is affectionately dedicated

PREFACE TO THE SECOND EDITION

The advancements that have taken place in all phases of bacteriology since this book was first published have made it necessary to prepare a second edition. In order to bring the material up to date, the book has been thoroughly revised and entirely rewritten.

To mention the chapters that have been revised would mean the inclusion of the entire book. However, the chapters that show the most significant changes are the following: The Morphology of Bacteria; The Microscope, including a brief discussion of the newer results and possibilities with the electron microscope; The Effect of Environment upon Bacteria; The Nutrition of Bacteria, including a discussion of the various growth factors or vitamins required by organisms; The Enzymes of Bacteria; the Respiration of Bacteria; The Fermentation of Carbohydrates and Related Compounds; Associations of Bacteria, including the newer work on bacterial antagonisms; Differentiation and Classification of Bacteria; Bacteriology of Air; Bacteriology of Soil; Bacterial and Virus Diseases of Plants; and Specific Infections.

The first edition contained a considerable amount of chemistry because it is believed that no student can intelligently understand or pursue research in bacteriology without first having had a sound knowledge of at least inorganic and organic chemistry. This feature of the first edition has been retained and perhaps emphasized to a greater extent in the present revision.

Considerably more textbook material has been incorporated in this edition. Because of this fact it was considered advisable to separate the laboratory procedures from the text material; otherwise, the book would have been too bulky. The experimental procedures have been incorporated into a laboratory manual to accompany the textbook. The separation of the laboratory material from the textbook will answer the objection of those instructors who prefer to use their own manuals.

The author has attempted to give credit for all illustrations and text material used in the book. Any omissions or errors are entirely unintentional. He wishes to thank his wife and Mitchell Korzenovsky and Harvey C. Upham for their aid in reading and checking the proof, and also those who have offered valuable suggestions during the preparation of this manuscript.

A. J. SALLE.

LOS ANGELES, CALIF.,
December, 1942.

PREFACE TO THE FIRST EDITION

"This is frankly a plea for the return of the dignity and importance of the preface. Too often the writing of a preface has become a chore, a necessary evil prescribed by custom. But like many practices which have become common through familiarity, the original purpose of the preface has perhaps been obscured by time and by the careless reading habits of the average reader who wishes to get to the meat of the book as quickly as possible. The preface is a vital part of the book and for good reasons. . . . "

"All of us come to a book loaded with prejudices. We are not as impartial as we think we are. Mention a topic or theme and we can be sure to express a certain point of view—right or wrong. It is the function of the preface to modify these prejudices by suggesting what presumably are new points of view. Thus, the preface is an exercise in persuasion. It must break down 'reader resistance'; it must put the reader in the proper frame of mind to approach the reading of the book. If the preface is written with this idea in mind, the reader will come to the book proper already favorably disposed toward the author. If the author is inclined to evade actualities, he must then be prepared for reader apathy and perhaps neglect. . . . ."

JOHN R. WILBUR.

This book has been written for those who are beginning the study of bacteriology and especially for those who plan to specialize in the subject. It is concerned chiefly with a discussion of the important principles and facts of bacteriology which a student should acquire in order to realize to the fullest extent the more advanced work on the subject.

The book is, as its name implies, a textbook on fundamentals. The author has tried at all times to keep this thought in mind in the preparation of the manuscript. The usual textbooks either are too elementary or do not contain sufficient fundamental material to give the beginning student a solid foundation on which to build for more specialized work on the subject. The author has tried to give explanations of all phenomena described in the book insofar as it is possible to do so, a point which has been greatly neglected in most texts. The book is profusely illustrated with chemical formulas because it is believed that no student can intelligently understand bacteriology without first having had at least inorganic and organic chemistry. This statement applies especially to the chapters on Biological Stains, Disinfection and Disinfectants, Enzymes of Bacteria, The Respiration of Bacteria, Protein Decomposition, Industrial Fermentations, The Bacteriology of Water, and The Bacteriology of Soil.

The book differs in one important respect from practically all texts on fundamental bacteriology in that it is written as a combination textbook and laboratory manual. The experimental portion is not added as an appendix but is woven into the body of the manuscript under the appropriate chapters. The textbook material goes hand in hand with systematically arranged laboratory procedures. It is believed that bacteriology cannot properly be understood or appreciated unless studied in conjunction with experimental laboratory work. The incorporation of laboratory exercises into the body of the book permits the reader better to understand the textbook material, and the addition of text to the laboratory portion aids the student better to understand the experimental procedures.

Sufficient experimental material has been included to meet the fundamental requirements of beginning students in the bacteriology major and of students in the various divisions of agriculture, forestry, home economics, sanitary engineering, physical education, hygiene, public health, etc. The number of experiments should prove ample for a one-semester course. The author has purposely included a large number in order that the instructor may make a selection if desired.

The names of the organisms used are those recommended by the Committee on Classification of the Society of American Bacteriologists. Although the system does not satisfy everyone, it comes nearer to being a standard classification than any that has been used before and it is now in general use in this country.

The author has attempted to indicate in the text the sources of the material and illustrations used. He wishes to thank all who have offered suggestions and have been of assistance in the preparation of the manuscript. He is especially indebted to his wife and to I. L. Shechmeister for their aid in reading and checking the proof. The author alone accepts responsibility for any defects that may be inherent in the plan and scope of the book and for errors that may have escaped detection.

A. J. SALLE.

BERKELEY, CALIFORNIA,
December, 1938.

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FUNDAMENTAL PRINCIPLES OF BACTERIOLOGY

CHAPTER I

INTRODUCTION

Bacteriology is the science that deals with the study of the organisms known as bacteria (singular, bacterium). Microbiology, in its broadest meaning is the science that deals with the study of all microorganisms, such as bacteria, yeasts, molds, and algae. The word germ is probably synonymous with bacterium. Although this book will include a discussion of microorganisms in general, the major portion of the material will be devoted to the study of bacterial organisms.

Man, who is forever classifying things, has placed living organisms into either the plant or the animal kingdom. Most living organisms possess the characteristics of one kingdom or the other and may be sharply differentiated. However, bacterial microorganisms display the characteristics of both plants and animals and, for this reason, it is not possible to place them in one group or the other.

Haeckel (1884) believed that considerable confusion could be avoided if the bacteria were placed in a new kingdom which he named the Protista. He grouped into this kingdom all microorganisms such as yeasts, molds, bacteria, protozoa, algae, which were placed with difficulty into the two older kingdoms. His suggestion did not gain wide acceptance and Haeckel finally abandoned the idea. After all, it makes little difference whether bacteria are plants or animals as long as their fundamental characteristics have been studied and are understood.

CHARACTERISTICS OF PLANTS AND ANIMALS

Bacteria are among the simplest forms of life known and hence show characteristics of both plants and animals. For the sake of convenience they have been grouped under the plant kingdom. According to Tanner (1937) some of the characters used to distinguish plants from animals are given in Table 1.

TABLE 1*

Plants	Animals
Store energy	Liberate energy
Cell walls composed of cellulose (carbohydrate)	Cell walls composed of nitrogenous compounds and carbohydrate
Root hairs and stomata absorb H_2O and gases. No digestion	Possess an alimentary canal in which digestion takes place
Take CO_2 and H_2O from the atmosphere and nitrogen salts from the soil to build up their proteins and carbohydrates. Oxygen liberated. Chemosynthetic	Cannot utilize CO_2 and H_2O from the atmosphere. Use organic compounds and liberate CO_2. Chemoanalytic
Vacuoles well-developed	Vacuoles absent or not well developed
Nucleoproteins contain a pentose	Nucleoproteins contain a desoxypentose
Do not have sensory organs or nervous system	Have sensory organs and nervous system

* Reprinted by permission from "Bacteriology—A Textbook of Microorganisms," by Tanner, published by John Wiley & Sons, Inc., New York, 1937.

A condensed classification of the plant kingdom is as follows:

CLASSIFICATION OF PLANTS

Phylum I. *Thallophyta.* The thallophytes or thallus plants do not have roots, stems, or leaves.

Subphylum 1. *Algae.* The algae possess the green coloring matter chlorophyll and are capable of manufacturing their own food from water and carbon dioxide of the atmosphere in the presence of sunlight.

Class I. *Diatomaceae,* the diatoms.
Class II. *Cyanophyceae,* the blue-green algae.
Class III. *Chlorophyceae,* the green algae.
Class IV. *Phaeophyceae,* the brown algae.
Class V. *Rhodophyceae,* the red algae.

Subphylum 2. *Fungi.* The fungi, with the possible exception of a few species, do not contain chlorophyll and are unable to synthesize their own food from water and carbon dioxide of the atmosphere. They must have their food materials in a preformed condition. In this group are placed the bacteria, molds, and yeasts.

Class I. *Schizomycetes,* the bacteria.
Class II. *Saccharomycetes,* the yeasts.
Class III. *Phycomycetes,* the alga-like fungi.
Class IV. *Ascomycetes,* the sac fungi.
Class V. *Basidiomycetes,* the basidia fungi.

Phylum II. *Bryophyta.* The bryophytes are the mosses.

Class I. *Hepaticae,* the liverworts.
Class II. *Muscineae,* the mosses.

Phylum III. *Pteridophyta.* The pteridophytes are the fern plants.

Class I. *Filicales,* the true ferns.
Class II. *Equisetales,* the horsetails.
Class III. *Lycopodiales,* the club mosses.

Phylum IV. *Spermatophyta.* The spermatophytes includes the seed plants.

Subphylum 1. *Gymnospermae,* the cone-bearing plants, pines, hemlocks, etc.

Subphylum 2. *Angiospermae,* the flowering plants.

Class I. *Monocotyledons,* endogenous plants.
Class II. *Dicotyledons,* exogenous plants.

The characteristics of the classes of the subphylum *Fungi*, which includes the bacteria, yeasts, and molds, are as follows:

Subphylum 2. *Fungi*. The thallus plants have neither roots, stems, nor leaves.

Class I. *Schizomycetes*. All the organisms are single-celled, with a possible few exceptions, contain no chlorophyll, and multiply normally by a process of transverse or binary fission. The cells may be spherical, cylindrical, comma-shaped, spiral, or filamentous and are often united into chains or into *flat* or cubical aggregates.

Class II. *Saccharomycetes*. The saccharomycetes include the yeasts. They are generally easily distinguishable from the bacteria in being larger and in having well-defined nucleuses. Yeasts multiply by budding, spore formation, fission, and by copulation, but usually by the process of budding.

Class III. *Phycomycetes*. The phycomycetes are filamentous alga-like fungi which do not form cross walls (nonseptate). Sexual spores are produced which are known as zygospores.

Class IV. *Ascomycetes*. The ascomycetes produce spores within sacs known as asci (singular, ascus). The spores are known as ascospores.

Class V. *Basidiomycetes*. The basidiomycetes reproduce by the formation of basidia. The mycelium is septate. Asexual spores and chlamydospores are also formed.

Class VI. *Fungi Imperfecti*. The fungi in this group are separated from the other fungi in not having well-defined fruiting bodies. The fungi that cannot be classified with the Phycomycetes, Ascomycetes, or Basidiomycetes are placed in this group. Some of the genera of the Fungi Imperfecti are *Oidium*, *Monilia*, *Endomyces*, *Torula*, *Mycoderma*, etc.

DISTRIBUTION OF BACTERIA

Bacteria are widely distributed in nature, being found nearly everywhere. They are found in the soil, air, water, foods, in decaying organic matter of all kinds, on the body surface, within the intestinal tract of man and animals, etc. The numbers vary from one place to another, depending upon the environmental conditions.

Some bacteria are more commonly distributed in certain places than others. The common occurrence of a species in a certain environment is spoken of as the natural flora of that particular environment. Changes in the environmental conditions produce changes in the bacterial flora.

Soil.—The numbers and kinds of organisms present in soils depend upon the kind of soil, quantity of plant and animal debris (humus), acidity or alkalinity, depth, moisture content, and treatment. The numbers decrease with depth, owing to lack of oxygen and food materials. A rich garden soil contains many more organisms than a poor uncultivated soil. The great majority of soil organisms are found in the surface layers.

Air.—Bacteria are found in the atmosphere, being carried there by air currents. Organisms do not grow and multiply in air because conditions are not favorable. There is no such thing as a normal atmospheric

flora. The numbers and kinds depend upon location, amount of moisture, dust particles, wind currents, and the presence of toxic gases. The air over the ocean far removed from shore is practically free from micro-organisms. The same holds true for air over high mountains. The air of city and country differ as to the numbers and kinds of species present. Dusty rooms usually show considerably more organisms than do rooms kept free from dust. Bacteria are usually found adhering to particles of dust. This means that the more particles suspended in air the greater will be the extent of bacterial contamination. Spores of yeasts, molds, and bacteria are commonly found in air owing to the fact that these bodies are more resistant to the ultraviolet rays of the sun than are the vegetative cells producing them. These bodies are a frequent cause of air contaminations in bacteriological laboratories and, because of their great resistance to heat, require high temperatures to destroy them.

Water.—Most waters contain large numbers of bacterial organisms. The numbers vary considerably, depending upon the source of the water, *e.g.*, from deep or shallow wells, springs, rivers, lakes, ponds, streams, etc. Water polluted with sewage may contain thousands or even millions of organisms per cubic centimeter. Under some conditions disease organisms may also be present. Some bacterial species are constantly present and constitute the natural flora of that water. Usually fewer bacterial species occur in sea water than in the soil. The absence of a high bacterial population in sea water is probably due to its poor qualities as a culture medium.

Foods.—Foodstuffs are rarely free from living organisms. Some of the organisms are of benefit in producing desirable fermentations, such as occur in the oxidation of alcohol to acetic acid or vinegar, the lactic fermentation of cabbage to sauerkraut, etc. Frequently undesirable organisms are found in foods and bring about abnormal changes. Sometimes foods are the cause of certain types of intoxications and disease processes due to the presence of pathogenic organisms.

Normal udders of cows are probably never free from bacteria, which means that freshly drawn milk is not sterile. The first milk to be drawn always contains more organisms than milk drawn at the close of the milking operation owing to the fact that the bacteria are washed away from the udders early in the process. However, most of the organisms found in milk are chiefly those which gain entrance during the operations of milking, handling, and storing. Unless the milk is stored at a low temperature immediately after collection, these organisms are capable of producing undesirable changes, making the milk unfit for human consumption.

Body.—The outer surface or skin of the body always contains micro-organisms. The same applies to the alimentary tract and respiratory

passages of man and animals. The skin, intestinal contents, and the respiratory passages contain a normal bacterial flora. These organisms are for the most part harmless. Occasionally some species penetrate the broken skin and intestinal wall, resulting in the establishment of a disease process. Usually the organisms are destroyed by the defense mechanisms of the host. It has been said that as much as one-fourth of the dry weight of the intestinal contents of man is composed of bacterial cells.

Escherichia coli is always found in the large intestine of man. There are other organisms present but in an adult on a mixed diet this organism predominates. The organism *E. coli*, then, is largely responsible for the natural flora of the large intestine. Changes in the environmental conditions produce changes in the bacterial flora. If the diet of an adult is changed from a high-protein to a high-carbohydrate diet the *E. coli* organisms will be gradually reduced in numbers only to be replaced by a much larger organism known as *Lactobacillus acidophilus*. If this particular diet is maintained *L. acidophilus* will now become the predominating organism of the large intestine.

FUNCTIONS OF BACTERIA

Those who are not familiar with the activities of bacteria usually believe that the vast majority of them are harmful; that their chief function in this world is to gain entrance to the body and produce various kinds of diseases. This statement is entirely erroneous. The great majority of the bacteria are not only harmless but absolutely necessary for the existence of living things. Life could not exist in the complete absence of bacteria. They are necessary for the disposal of human and animal carcasses. The remains of plant crops, plant stubble, leaves, etc., are converted into soluble compounds by the soil organisms and made available to the new plants. Some species are capable of taking nitrogen from the air and converting it into compounds that are utilized by the plants. In the absence of fertilizers such as animal manures, nitrates, and ammonium salts, there would be no nitrogen in the soil were it not for the activities of these organisms. Sulfur and phosphorus, two necessary elements for plant growth, are also converted into soluble inorganic compounds and absorbed by plant roots.

Fertile soils may always be distinguished from poor soils in containing greater numbers of viable organisms. If the soil is rich in plant remains, contains sufficient moisture, and shows the right temperature and hydrogen-ion concentration (reaction), many organisms will be present to attack the plant and animal residues, converting the insoluble and indiffusible constituents into soluble, diffusible compounds utilizable by the plants.

Bacteria are necessary for the disposal of sewage. They convert the insoluble proteins, fats, carbohydrates (cellulose) into soluble odorless compounds, which may be disposed of in an inoffensive manner.

The souring of milk is the result of bacterial action. This is the first step in the preparation of butter and various types of cheeses. The ripening of cheese is brought about by the action of bacteria and molds, which are responsible for the odors and flavors imparted to cheeses.

These are only a few examples of the part played by the associated activities of organisms in nature. Many other useful purposes will be discussed under the various chapters in this book.

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CHAPTER II

THE MICROSCOPE

The compound microscope may be defined as an optical instrument consisting of a combination of lenses for making enlarged or magnified images of minute objects. The term is compounded from the two Greek words, μῖκρός, *micro*, small, and σκοπεῖν, *scope*, to view.

Bacteria are so small that they cannot be seen with the naked eye. They must be greatly magnified before they can be clearly seen and studied. The use of a microscope is, therefore, absolutely indispensable to the bacteriologist and to the biologist in general.

The student should first understand the principles involved in order that the microscope may be employed to the greatest advantage. As Sir A. E. Wright (1907) stated,

> Every one who has to use the microscope must decide for himself the question as to whether he will do so in accordance with a system of rule of thumb, or whether he will seek to supersede this by a system of reasoned action based upon a study of his instrument and a consideration of the scientific principles of microscopical technique.

GENERAL PRINCIPLES OF OPTICS

A simple microscope, or a single microscope, consists merely of a single lens or magnifying glass held in a frame, usually adjustable, and often provided with a stand for conveniently holding the object to be viewed and a mirror for reflecting the light. A compound microscope differs from a simple microscope in that it consists of two sets of lenses, one known as an objective and the other as an eyepiece, commonly mounted in a holder known as a body tube (Fig. 1). Accurate focusing is attained by a special screw appliance known as a fine adjustment. Compound microscopes give much greater magnifications than simple microscopes and are necessary for viewing and examining such minute objects as bacteria.

The path of light through a microscope is illustrated in Fig. 2. The light, in passing through the condenser, object in Plane I, and objective lens would form a real and inverted image in Plane II if the ocular or eyepiece were removed. In the presence of the ocular *F* the rays are intercepted, forming the image in Plane III. The real image is then examined with the eye lens *E* of the ocular acting as a single magnifier

and forming a virtual image in Plane IV. The distance between the virtual image (Plane IV) and the eyepoint is known as the projection distance. The object is magnified first by the objective lens and second

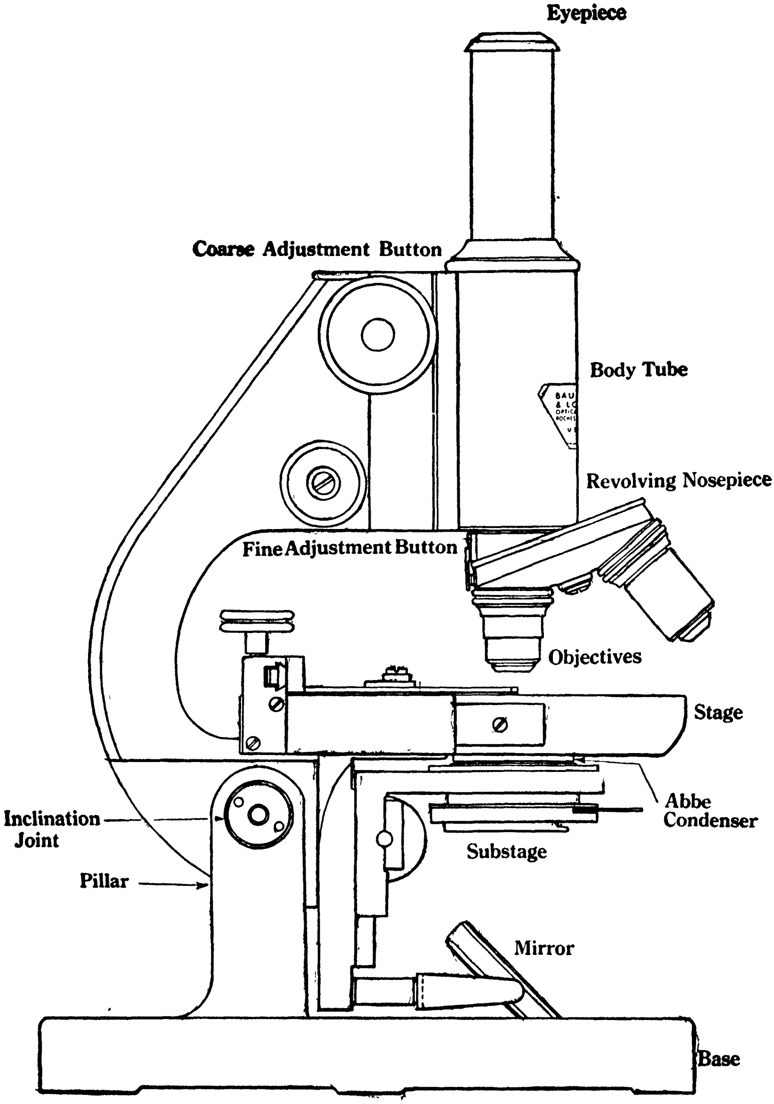


FIG. 1.—Compound microscope and its parts. (*Courtesy of Bausch and Lomb Optical Company.*)

by the ocular or eyepiece. With a tube length of 160 mm. (most microscope manufacturers have adopted 160 mm. as the standard tube length), the total magnification of the microscope is equal to the magnifying

power of the objective lens multiplied by the magnifying power of the ocular.

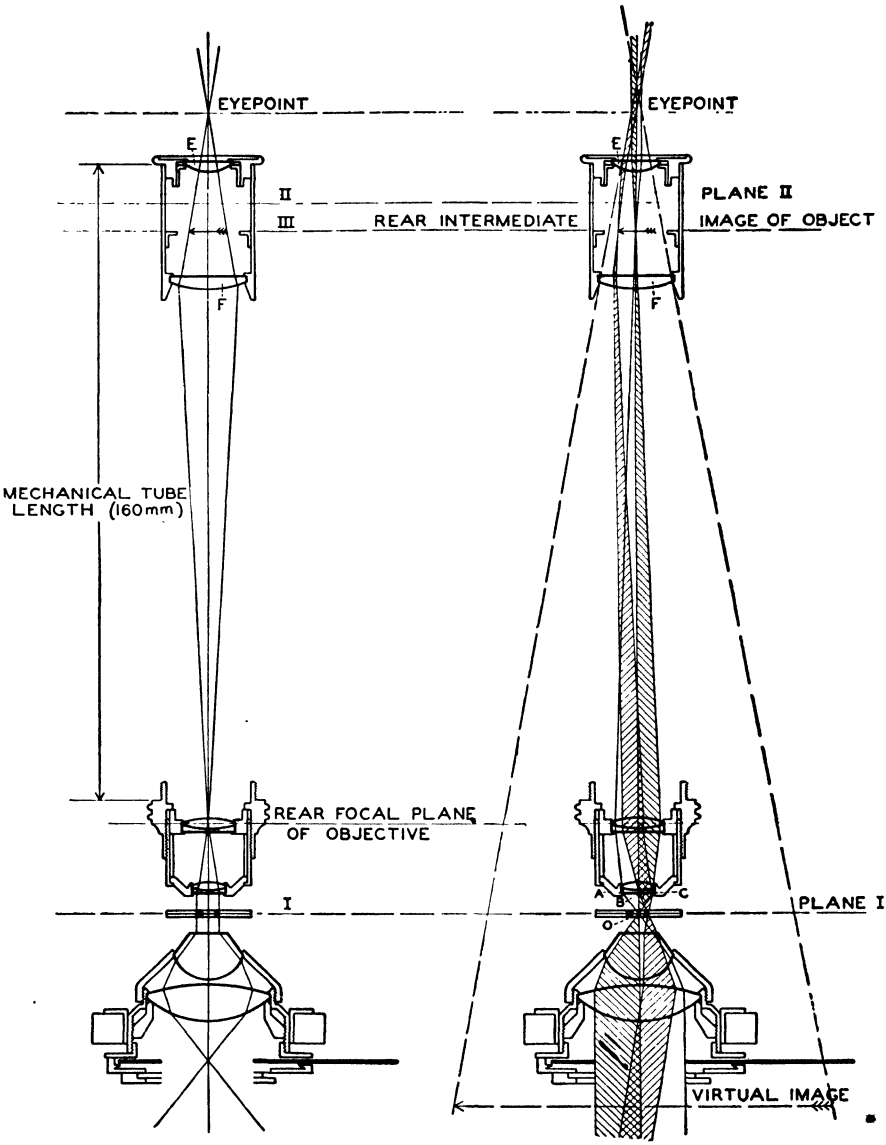


FIG. 2.—Path of light through a microscope. (*From Photomicrography, courtesy of Eastman Kodak Company.*)

The above magnifications are obtained on a ground glass placed 10 in. from the ocular of the microscope. After the microscope has been set at the proper tube length, the total magnification may be computed by

multiplying the magnifying power of the objective by that of the eyepiece and by one-tenth of the distance from the eyepiece to the ground glass measured in inches. For example, if the ground glass is placed 10 in. from the eyepiece of the microscope the total magnification will be as given on the ocular and objective. If the ground glass is placed 20 in. from the eyepiece the magnification will be twice as great. If placed 5 in. from the eyepiece the magnification will be one-half as great. To take a specific example:

Magnification of objective..........	97×
Magnification of ocular............	10×
Distance of ground glass from ocular	7 in.
Total magnification..............	97 × 10 × 0.7(0.1 × 7) = 679×

It may be seen that almost any degree of magnification could be obtained by using oculars of different magnifying powers or by varying the length of the draw tube. Even though the magnifying powers of the microscope could be greatly increased in this manner, the amount of detail that can be seen is not improved since this is strictly limited by the structure of light.

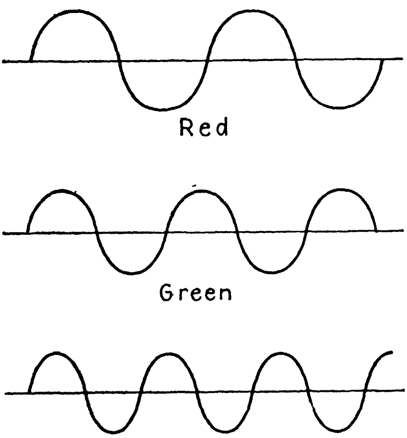


FIG. 3.—Wave lengths of light of different colors.

Structure of Light.—It is generally agreed that light is transmitted from luminous bodies to the eye and other objects by the undulating or vibrational movement of the ether. This is known as the undulatory or wave theory of light. Light waves travel at the rate of about 186,300 miles per second and the vibrations are transverse to the direction of the propagation of the wave motion.

When a beam of white light is passed through a prism, a spectrum is obtained in which several colors form a series from deep red through orange, yellow, green, blue, and indigo to deepest violet. It is known that the wave lengths of the various colors are different, that red shows the longest and violet the shortest waves of the visible spectrum.

The length of a light wave is the distance from the crest of one wave to the crest of the next (Fig. 3). The unit of measurement is the angstrom unit (Å.) which is equal to 1/10,000,000 mm. or to approximately 1/250,000,000 in. The visible spectrum, together with the corresponding wave lengths of the light rays in angstrom units, may be represented

as shown in Fig. 4. Visible light waves, ranging in length from 4000 to 7000 Å., may be roughly divided into three portions: blue violet, from 4000 to 5000 Å.; green, from 5000 to 6000 Å.; red, from 6000 to 7000 Å.

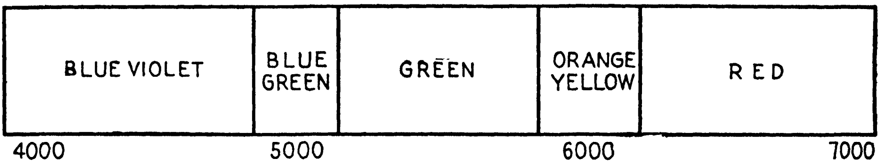


FIG. 4.—Light rays of the visible spectrum and their corresponding wave lengths in angstrom units.

OBJECTIVES

The objective is the most important lens on a microscope because its properties may make or mar the final image. The chief functions of the objective lens are (1) to gather the light rays coming from any point of the object, (2) to unite the light in a point of the image, and (3) to magnify the image.

Numerical Aperture.—The resolving power of an objective may be defined as its ability to separate distinctly two small elements in the structure of an object, which are a short distance apart. The measure for the resolving powers of an objective is the numerical aperture (N.A.). The larger the numerical aperture the greater the resolving power of the objective and the finer the detail it can reveal.

Since the limit of detail or resolving power of an objective is fixed by the structure of light, objects smaller than the smallest wave length of visible light cannot be seen. In order to see such minute objects it would be necessary to use rays of shorter wave length. Invisible rays, such as ultraviolet light, are shorter than visible rays but, since they cannot be used for visual observation (photography only), their usefulness is limited.

The image of an object formed by the passage of light through a microscope will not be a point but, in consequence of the diffraction of the light at the diaphragm, will take the form of a bright disk surrounded by concentric dark and light rings (Fig. 5). The brightness of the central disk will be greatest in the center, diminishing rapidly toward the edge. The image cone of light composed of a bright disk surrounded by concentric dark and light rings is spoken of as the antipoint. If two independent points in the object are equidistant from the microscope lens, each will produce a disk image with its surrounding series of concentric dark and light rings. The disks will be clearly visible if completely separated but, if the images overlap, they will merge into a single bright area the central portion of which appears quite uniform. The two disks will not, therefore, be seen as separate images. It is not definitely known

just how close the centers of the images can be and still allow them to be seen as separate antipoints.

The minimum distance between the images of two distinct object points decreases as the angle of light *AOC* (Fig. 2), coming from the object *O*, increases. The angle formed by the extreme rays is known as the aperture of the objective. The ability of the objective lens system to

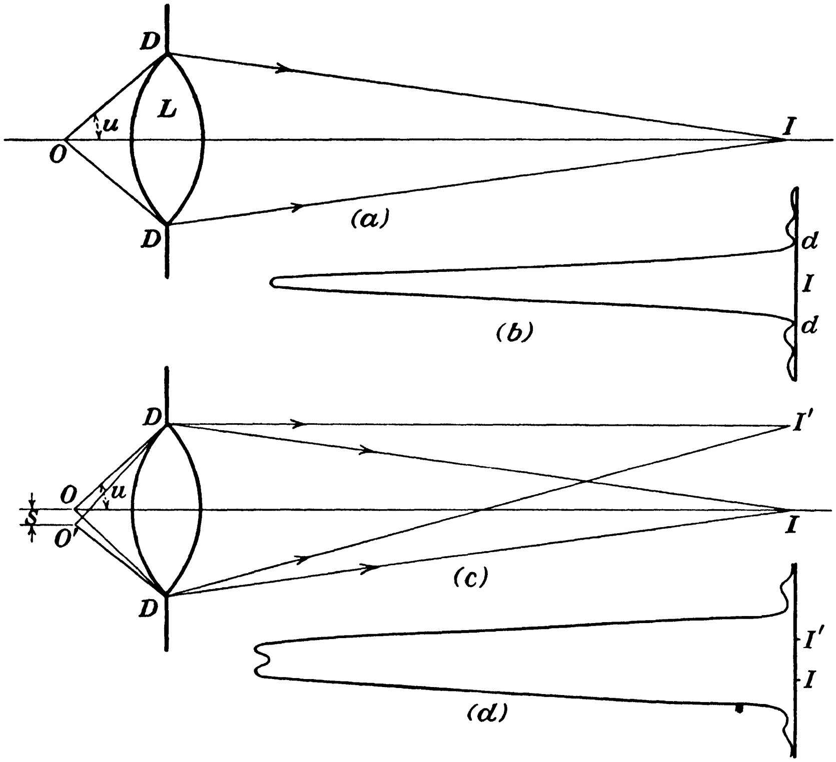


FIG. 5.—Resolving power of an objective. (*a*) The rays from the object at *O* form an image at *I*. (*b*) Distribution of light in the image at *I*. The bright disk, *dd*, is surrounded by concentric dark and light rings. (*c*) Two independent points in the object *O* and *O'*, form two images at *I* and *I'*. (*d*) The two independent object points *O* and *O'* are so close together that their images overlap at *I* and *I'* and merge into a single bright area, the central portion of which appears quite uniform. (*From Sir Herbert Jackson and H. Moore, Microscope, courtesy of the Encyclopaedia Britannica, Inc.*)

form distinct images of two separate object points is proportional to the trigonometric sine of the angle. The latter, then, is a measure of the resolving power of the objective. Actually, however, the sine of angle *AOB* is used, which is just one-half of angle *AOC*. This is usually referred to as sin u. Since the sine of an angle may be defined as the ratio of the side opposite the angle in a right-angled triangle to the hypotenuse then,

$$\sin u = \frac{AB}{AO}$$

The light, in passing through the objective, is influenced by the refractive index n of the space directly in front of the lens. This is another factor that affects the resolving power of an objective. The two factors, refractive index n and $\sin u$, may be combined into a single expression known as the numerical aperture, which may be expressed as follows:

$$\text{N.A.} = n \sin u$$

Importance of N.A.—If a very narrow pencil of light is used for illumination, the finest detail that can be revealed by a microscope with sufficient magnification is equal to

$$\frac{\text{w.l.}}{\text{N.A.}}$$

where w.l. is the wave length of the light used for illumination and N.A. is the numerical aperture of the objective. The resolving power of the objective is proportional to the width of the pencil of light used for illumination. This means that the wider the pencil of light the greater the resolving power. The maximum is reached when the whole aperture of the objective is filled with light. In this instance the resolving power is twice as great. The finest detail that the objective can reveal is now equal to

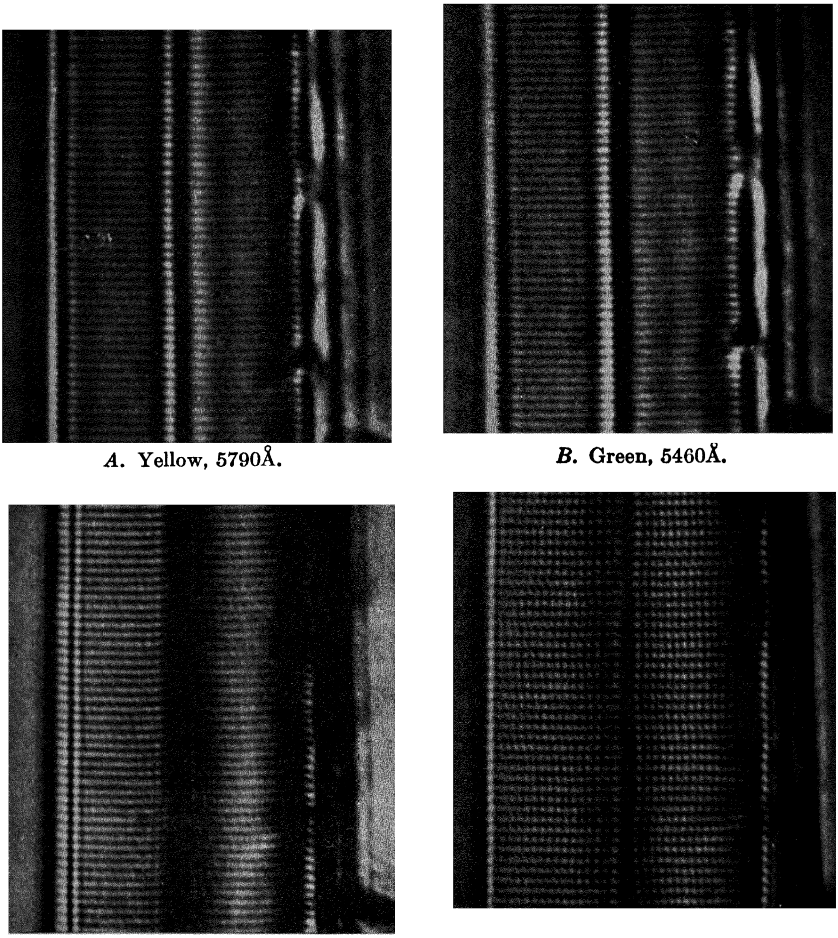
$$\frac{\text{w.l.}}{2\text{N.A.}}$$

For example, the brightest part of the spectrum shows a wave length of 5300 Å. An objective having a numerical aperture equal to 1.00 will resolve two lines separated by a distance of 5300 Å./1.00 = 5300 Å. (48,000 lines to the inch) if a very narrow pencil of light is used, and 5300 Å./(2 × 1.00) = 2650 Å. (95,000 lines to the inch) if the whole aperture of the objective is filled with light.

From the above it is evident that the maximum efficiency of an objective is not reached unless the back lens is filled with light. This may be ascertained by removing the eyepiece from the microscope and viewing the back lens of the objective with the naked eye. If the back lens is completely filled with light, the efficiency will then be according to the numbers engraved on the objective.

Resolving Power.—The relation between wave length and resolving power is illustrated in Fig. 6. The shorter the wave length of light the finer the detail revealed by the objective. Using an objective having a N.A. of 1.00 and a yellow filter (light transmission of 5790 to 5770 Å.) it is possible to see about 88,000 lines to an inch; with a green filter (light

transmission of 5460 Å.) about 95,000 lines to an inch; with a violet filter (light transmission of 4360 Å.) about 115,000 lines to an inch; and with ultraviolet light (light transmission of 3650 Å.) about 140,000 lines to an inch.



A. Yellow, 5790Å. *B.* Green, 5460Å.

C. Violet, 4360Å. *D.* Ultraviolet, 3650Å.

FIG. 6.—*Amphipleura pellucida*, a diatom. Effect of light of different wave lengths on the resolving power of the objective. (*From Photomicrography, courtesy of the Eastman Kodak Company.*)

Electron Microscope.—Within the last few years a new type of instrument has been developed having a higher resolving power than the usual microscope employing visible light. This instrument employs a beam of electrons, instead of visible light rays. It is known as the electron microscope (Fig. 7).

Beams of electrons or cathode rays (1) travel in a straight line in the absence of any matter or field, (2) can be concentrated in certain cases, and (3) can be deflected in an electrostatic or magnetic field. A close analogy exists between the action of a magnetic or electric field of rotational symmetry on an electron beam and the action of a glass lens on a light beam (Fig. 8*A*, *B*).

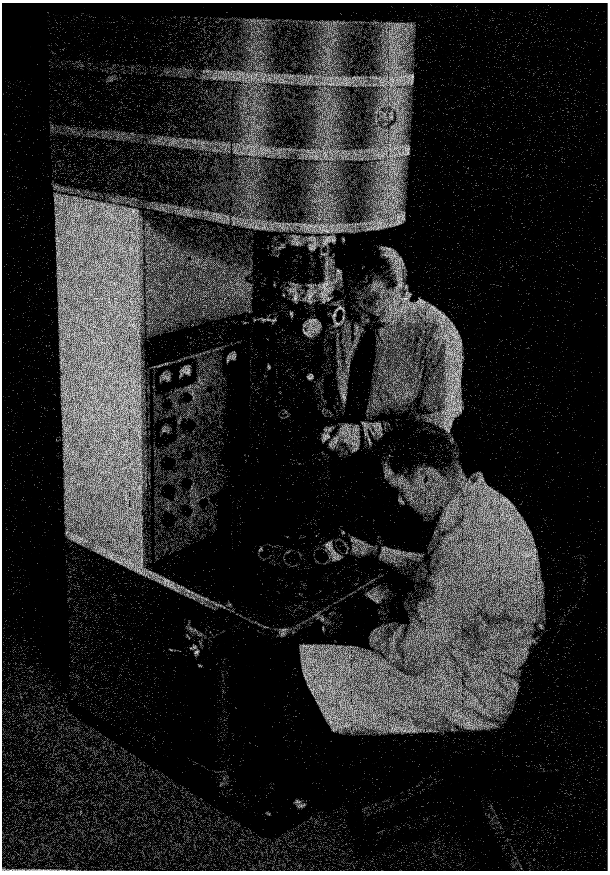


FIG. 7.—RCA electron microscope and its associated power supply. (*From Electron Microscope, courtesy of RCA Manufacturing Company, Inc.*)

In the electron microscope it is possible to make highly magnified images with electron optical systems by combining two or more electronic "lenses." The wave length of the electron is considerably smaller than that of visible light, being a function of the electron velocity. For velocities between 30,000 and 100,000 volts the wave length is only 1/100,000 of the wave length of visible light. The resolving power is

so much greater than that of the ordinary microscope employing visible light that it is now possible to obtain images of particles of atomic dimensions. If the best microscope employing visible light gives a magnification of 2000 diameters, the electron microscope gives 50 times the resolving power or a magnification of about 100,000 diameters. There is no doubt that further research will give even greater magnifications. It is now possible to see certain protein molecules, virus crystals,

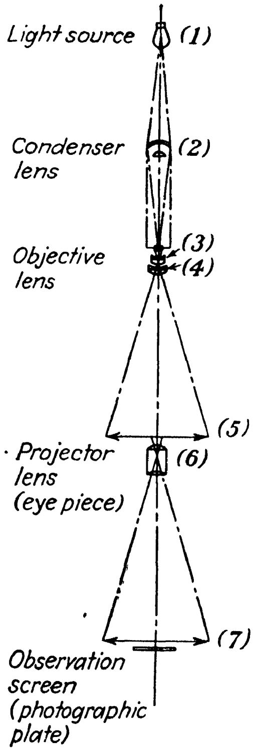


FIG. 8*A*.—Schematic diagram of light microscope. (*After Hillier and Vance.*)

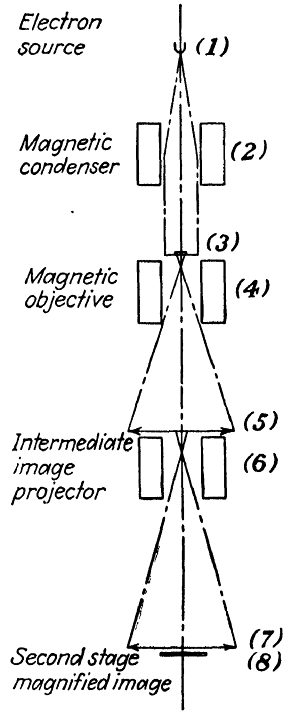


FIG. 8*B*.—Simplified schematic cross section of magnetic electron microscope. (*After Hillier and Vance.*)

unstained flagella, internal structures of bacteria, etc. (Figs. 9 and 10). For additional information see the excellent discussion by Hillier and Vance (1941).

Immersion Objectives.—When a dry objective is used, an air space is present on both sides of the microscope slide and cover slip. The largest cone of light that could possibly be used is 180 deg. in air, which is equal to an angle of about 82 deg. in the glass. This corresponds to a numerical aperture of 1.0. In actual practice, however, these figures become 143

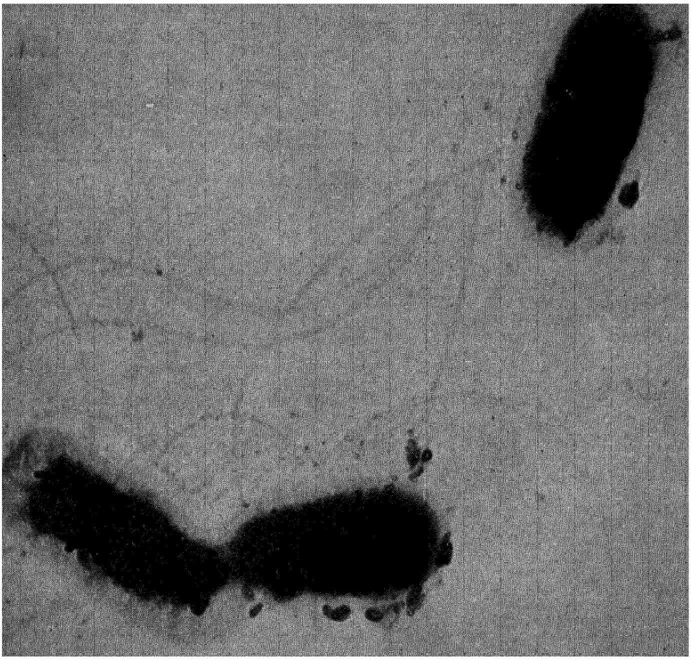


FIG. 9.—*Aerobacter cloacae*, showing unstained flagella. Magnified 27,000 times. *(From Electron Microscope, courtesy of RCA Manufacturing Company, Inc.)*

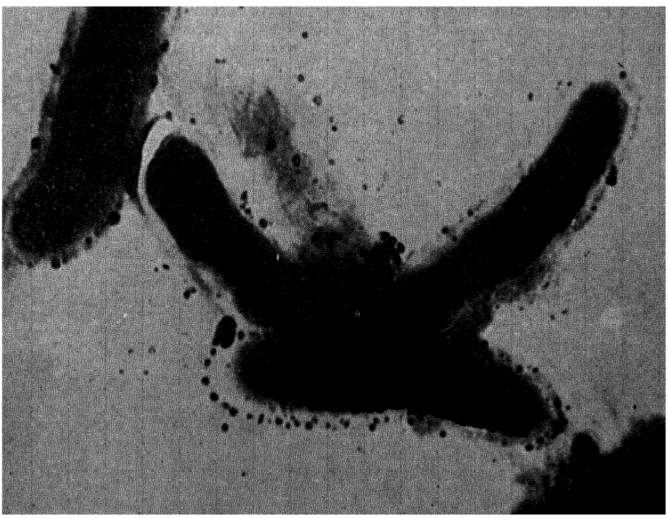


FIG. 10.—*Mycobacterium tuberculosis*. Magnified 21,000 times. *(From Electron Microscope, courtesy of RCA Manufacturing Company, Inc.)*

and 77, respectively, owing to the fact that the air space must be wide enough to correspond to a practical working distance of the objective (Fig. 11). Rays of greater angular aperture than 82 deg. in glass, which originate at the object point O by diffraction will be completely reflected at the upper surface of the coverslip, t.

The refractive index n of the air is equal to 1.0. If the air space between the cover slip and the objective is filled with a fluid having a higher refractive index, such as water (n = 1.33) or, what is still better, a liquid having a refractive index approaching that of glass, such as cedarwood oil (n = 1.51), angles greater than 82 deg. are obtained. Numerical apertures greater than 1.0 are realized by this method. Cedarwood oil causes the light ray to pass right through the homogeneous medium with the result that a cone of light of about 134 deg. is obtained,

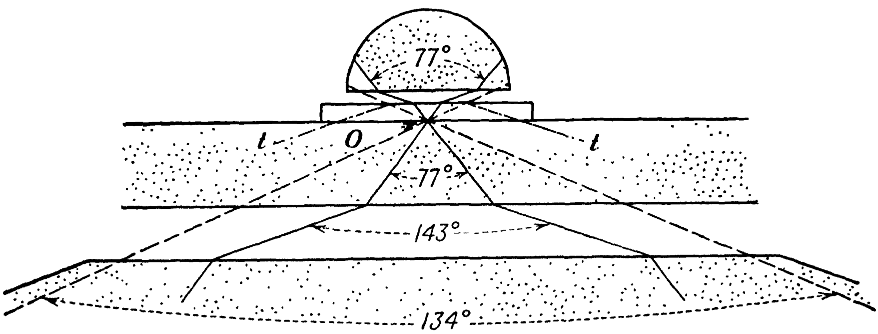


FIG. 11.—Passage of light through an object on a glass slide using dry and immersion objectives. See text for details. (*Redrawn from Photomicrography, Eastman Kodak Company.*)

which corresponds to a numerical aperture of 1.4. Finer detail can, therefore, be resolved by this procedure. With an oil immersion objective and a numerical aperture of 1.4, two lines as close together as 1/100,000 in. (0.2 μ) can be separated. This means, then, that the greater the numerical aperture of the objective the greater will be its resolving power or ability to record fine detail.

The refractive indexes of a number of media, which have been employed for immersion objectives, are given in Table 2.

TABLE 2

Medium	Refractive Index at 25°C.
Water	1.33
Glycerol	1.46
Mineral (paraffin) oil	1.47
Cedarwood oil	1.51
Sandalwood oil	1.51
Balsam	1.53

Depth of Focus.—The depth of focus is known also as the depth of sharpness or penetration. The depth of focus of an objective depends upon the N.A. and the magnification, and is inversely proportional to both. This means that the higher the N.A. and the magnification the less the depth of focus. Therefore, high-power objectives must be more carefully focused than low-power objectives. These conditions cannot be changed by the optician.

Equivalent Focus.—Objectives are sometimes designated by their equivalent focal length measured in either inches or millimeters. An objective designated by an equivalent focus of $\frac{1}{12}$ in. or 2 mm. means that the lens system produces a real image of the object of the same size as is produced by a simple biconvex or converging lens having a focal distance of $\frac{1}{12}$ in. or 2 mm. An objective designated 1.3 in. or 33 mm. produces a real image of the same size as is produced by a simple biconvex or converging lens having a focal distance of 1.3 in. or 33 mm.

Working Distance of Uncovered Objects.—If the object on a glass slide is not covered with a cover slip, the working distance may be defined as the distance between the front lens of the objective and the object on the slide when in sharp focus. The working distance is always less than the equivalent focus of the objective. This is illustrated in Fig. 12*A*. The working distance may be determined easily by noting the number of complete turns of the micrometer screw (fine adjustment) required to raise the objective from the surface of the slide where the object is located to a point where the microscope is in sharp focus.

To take a specific example:
Each turn of the micrometer screw = 0.1 mm.
Number of turns required to bring object in sharp focus = 6
Then,

$$\text{Working distance} = 6 \times 0.1 = 0.6 \text{ mm.}$$

Working Distance of Covered Objects.—If the object is covered with a cover slip, the free distance from the upper surface of the cover slip to the front of the objective will be less than in the case of an uncovered object. It is obvious from this that if the cover glass is thicker than the working distance of the objective, it will be impossible to get the object in focus. On the other hand if the glass is thin, it will be possible to get the object in focus but the focus of the microscope on a covered object will be different from that on an uncovered object. It follows from this that an object covered with a glass cover slip or other highly refractive body will appear as if it is raised and the amount of elevation will depend upon the refractive index of the glass or other medium covering the object. Also, the greater the refraction of the covering body the more will be the apparent elevation. This is shown in Fig. 12*B*, *C*. The apparent depth

of the object below the surface of the covering medium may be calculated by taking the reciprocal of its index of refraction. For example, if a glass cover slip is used, it will have an index of refraction of 1.52. The reciprocal of this figure is $1/1.52 = \frac{2}{3}$, approximately. This means that the apparent depth of the object is only two-thirds its actual depth.

The working distance of covered objects may be determined by noting the number of complete turns of the micrometer screw (fine adjustment)

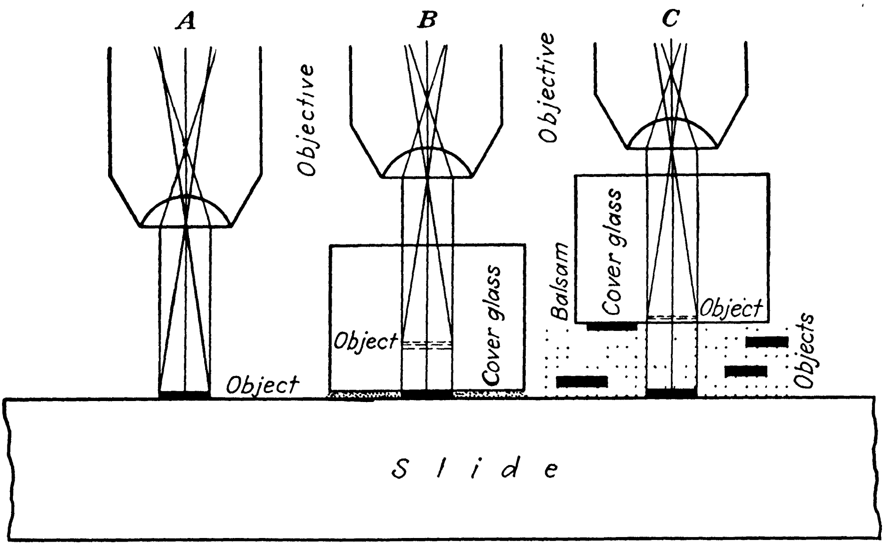


FIG. 12.—Working distance of an objective. *A*, object not covered with a cover glass. *B*, *C*, object covered with a cover glass. *(Redrawn from Gage, The Microscope, The Comstock Publishing Company, Inc.)*

required to raise the objective from the surface of the cover slip to a point where the objective is in sharp focus.

To take a specific example:
Each turn of micrometer screw = 0.1 mm.
Number of turns required to bring object in sharp focus = 3.5
Then,

$$\text{Working distance} = 3.5 \times 0.1 = 0.35 \text{ mm.}$$

Chromatic Aberrations in Objectives.—As has already been stated (page 10), white light, in passing through a prism, is broken up into its constituent colors, the wave lengths of which are different. A simple or compound lens, composed of only one material, will exhibit different focal lengths for the various constituents of white light. This is due to the dispersive power of the lens. Every wave length is differently refracted, the shortest waves most and the longest waves least. The blue-violet rays cross the lens axis first and the red rays last. There

will be a series of colored foci of the various constituents of white light extending along the axis. This is shown in Fig. 13. The lens will not therefore produce a sharp image with white light. Instead, the image will be surrounded by colored zones or halos, which interfere with the visual observation of its true color. This is spoken of as chromatic aberration. It may be lessened by reducing the aperture of the lens or, better still, by using a lens composed of more than one material (compound lens). Two or more different glasses or minerals are necessary for correcting the chromatic aberration of an objective and the amount of correction depends upon the dispersive powers of the components of the objective.

If two optical glasses are carefully selected to image light of two different wave lengths at the same focal point, the lens is said to be achromatic and an objective containing such a lens system is spoken

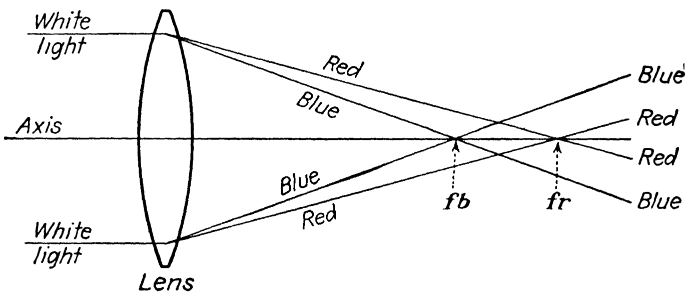


FIG. 13.—Chromatic aberration with white light. White light, in passing through a lens, is dispersed into its constituent colors. The red or long waves are refracted less than the blue or short waves. The blue rays (*fb*) cross the optical axis of the lens before the red rays (*fr*). The blue light will focus nearer the lens than the red light.

of as an achromatic objective. The remaining rays of the white light will be imaged at approximately the same point. An achromatic objective will yield images free from pronounced color halos. If the focus is shifted slightly, faint green and pink halos may be observed. The slight residual color will not prove objectionable for the usual microscopic work. Achromats are the universal objectives for visual work and are very satisfactory in photomicrography when used in monochromatic light (obtained by the use of filters).

Lens systems corrected for light of three different wave lengths are called apochromatic objectives. These objectives are composed of fluorite in combination with lenses of optical glass. The images produced by objectives in this group exhibit only a faint blue or yellow residual color. Since these objectives are corrected for three colors instead of for two, they are superior to the achromats. Their finer color correction makes possible a greater usable numerical aperture. The violet rays are brought to the same focus as visual rays. This fact makes these

objectives excellent for photographic use for both white and monochromatic light.

Another group of objectives exhibit qualities intermediate between the achromats and the apochromats. These are called semiapochromats. If the mineral fluorite is used in their construction, they are termed fluorite objectives. These objectives also yield excellent results when used for photomicrography.

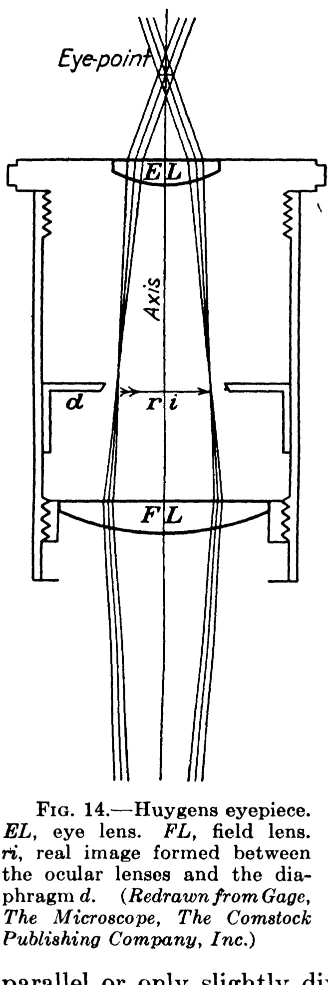


FIG. 14.—Huygens eyepiece. *EL*, eye lens. *FL*, field lens. *ri*, real image formed between the ocular lenses and the diaphragm *d*. (*Redrawn from Gage, The Microscope, The Comstock Publishing Company, Inc.*)

OCULARS

The chief functions of the ocular or eyepiece are the following:

1. It magnifies the real image of the object as formed by the objective.
2. It corrects some of the defects of the objective.
3. It images cross hairs, scales, or other objects located in the eyepiece.

Several types of eyepieces are employed, depending upon the type of objective located on the microscope. Those most commonly used are known as Huygenian, compensating, and hyperplane oculars.

Huygens Eyepiece.—In this type of eyepiece two simple plano-convex lenses are employed one of which is below the image plane (Fig. 14). The convex surfaces of both lenses face downward. Oculars in this group are sometimes spoken of as negative eyepieces. This type of ocular is made with a large field lens, which bends the pencils of light coming from the objective towards the axis without altering to any great extent the convergence or divergence of the rays in the individual pencils. Above the field lens and at some distance from it is a smaller lens known as the eye lens, the function of which is to convert each pencil of light into a parallel or only slightly diverging ray system capable of being focused by the eye. The rays, after emerging through this lens, then pass through a small circular area known as the Ramsden disk or eyepoint. It may be seen that the real image of the object is formed between the two eyepiece lenses. In an eyepiece of this type the distance separating the two lenses is always a little greater than the focal length of the eye lens. The reason for this is to prevent any dirt on the field lens from being seen

sharply focused by the eye. An image should be viewed with the eye placed at the Ramsden disk in order to obtain the largest field of view and, also, to obtain the maximum brightness over the field.

A Huygenian eyepiece should give equal magnification of light of different colors even though the images given by the various rays do not lie in the same plane. This is due to the fact that the eye is not very sensitive to off-focus effects. The eyepiece is practically achromatic. Huygenian eyepieces are intended to be used primarily with achromatic objectives.

Compensating Eyepiece.—Oculars of this type consist of an achromatic triplet combination of lenses (Fig. 15). These eyepieces are more perfectly corrected than are those of the Huygenian type. A compensating eyepiece is corrected to neutralize the chromatic difference of magnification of the apochromatic objectives. Such eyepieces are intended, therefore, to be used primarily with apochromatic objectives, although they may be employed successfully with the higher power achromats and fluorite objectives with good results.

Flat-field Eyepieces.—Apochromatic objectives, when used with compensating eyepieces, give fields that are not flat. Flat-field eyepieces have been designed to correct this defect. They give much flatter fields than do the other types already discussed, but they are less perfectly corrected chromatically. Oculars of this type are referred to as hyperplane, planoscopic, periplan, etc. They may be employed with the higher power achromatic, fluorite, and apochromatic objectives without introducing chromatic aberrations into the image. Their color compensation falls about midway between the Huygenian and compensating eyepieces.

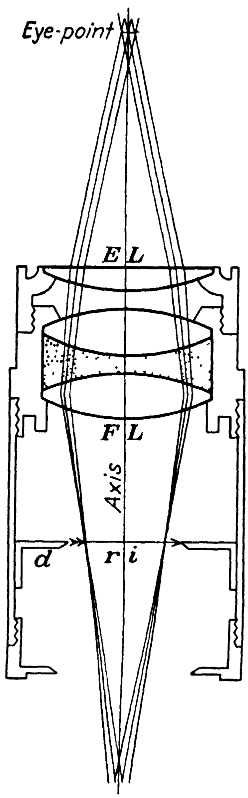


FIG. 15.—Compensating eyepiece. *EL*, eye lens. *FL*, field lens composed of three components. *ri*, real image formed below the lenses at the diaphragm *d*. (*Redrawn from Gage, The Microscope, The Comstock Publishing Company, Inc.*)

CONDENSERS

A condenser may be defined as a series of lenses for illuminating, with transmitted light, the object to be studied on the microscope. It is located under the stage of the microscope between the mirror and the object, whereas the objective and ocular lenses are located above the stage. It is sometimes referred to as a substage condenser (Fig. 16).

A condenser is necessary for the examination of an object with an oil-immersion objective in order to obtain adequate illumination. A condenser is also preferable when working with high-power dry objectives. Probably the most commonly employed condenser has a numerical aperture of 1.2. If an immersion objective having a numerical aperture of 1.3 is used, the condenser should have a numerical aperture of 1.4. As the numerical aperture of a condenser increases, the working distance and also the area illuminated decrease.

A good condenser sends light through the object under an angle sufficiently large to fill the aperture of the back lens of the objective. When this is accomplished the objective will then show its highest numerical aperture. This may be determined by first focusing the oil-immersion objective on the object. The eyepiece is then removed from the ocular tube. The back lens of the objective is observed by looking

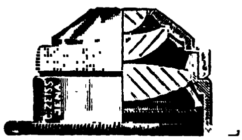


FIG. 16.—Substage condenser. N.A. = 1.4. (*Courtesy of Carl Zeiss, Inc.*)

down the microscope tube, care being taken not to disturb the focus. The back lens of the objective should be evenly illuminated. If it is not, the mirror should be properly centered. If the condenser has a smaller numerical aperture than the objective, the peripheral portion of the back lens of the objective will not be illuminated, even though the condenser iris diaphragm is wide open. If the condenser has a greater numerical aperture than that of the objective, the back lens of the objective may receive too much light, resulting in a decrease in contrast. The smaller the aperture the greater the depth of focus and the greater the contrast of the components of the image. The lowest permissible aperture is reached when diffraction bands become evident about the border of the object imaged. This difficulty may be largely overcome by closing the iris diaphragm of the condenser until the leaves of the iris appear around the edges of the back lens of the objective. The diaphragm is said, then, to be properly set. The setting of the iris diaphragm will vary with different objectives.

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CHAPTER III

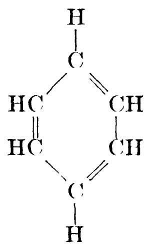
BIOLOGICAL STAINS

GENERAL CONSIDERATIONS

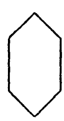
Bacteria are almost colorless and for this reason are difficult to see when viewed under the microscope. In order to be clearly seen they must first be stained. Staining bacterial cells is also important in revealing their structure. The presence of certain structures in their protoplasm furnishes the basis for the classification of some organisms. Without first staining, such bodies could not be seen.

Natural dyes predominated during the early years of bacteriology, but at present only a few of them are being used. They have been gradually replaced by the artificial or synthetic dyes. Since the first artificial dyes were produced from aniline, they are generally referred to as aniline dyes. However, there are a large number of them that are not derived from aniline and bear no relation to that compound. The term is now being replaced by the more accurate expression "coal-tar dyes," since all of them are derived from one or more substances found in coal tar.

The coal-tar dyes may be considered as derivatives of the cyclic compound benzene or benzole:

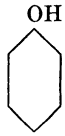


The empirical formula is C_6H_6. It is customary to write the structural formula by omitting the double bonds and the hydrogen atoms, abbreviating it to a hexagon, each corner of which represents an atom of carbon and one of hydrogen:

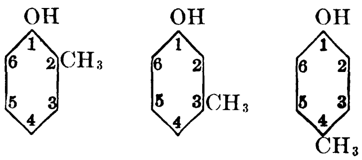


This hexagon is known as the benzene ring.

One or more hydrogen atoms may be replaced by some element or radical. For example, if one hydrogen atom is replaced by a hydroxyl (OH) group, the compound phenol or carbolic acid (C_6H_5OH) is produced.

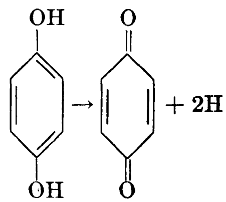


If another hydrogen atom is replaced by a methyl group (CH_3), the compound known as cresol is produced. Three different cresols are possible, depending upon which hydrogen atoms are substituted. The three possible cresols are as follows:



The substituted radicals are in the 1–2 or ortho; 1–3 or meta; and 1–4 or para positions. The compounds are named orthocresol, metacresol, and paracresol, respectively. The prefixes are usually abbreviated to the letters *o*-, *m*-, and *p*-.

The quinones are compounds derived by the elimination of two hydroxyl-hydrogen atoms from aromatic di-hydroxy derivatives. The simplest quinone is benzoquinone. It is also called quinone.



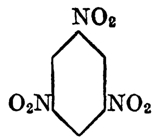
The benzene ring of the quinones contains two double bonds instead of three as in cresol. The formula of benzoquinone shows that it is not a true benzene derivative but the diketone of a *p*-hydrobenzene. Substances containing the quinone ring are called quinoid compounds. The double bonds in the quinoid compounds are supposed to be fixed, not mobile as in benzene. A large number of dyes contain the quinone ring.

DYES

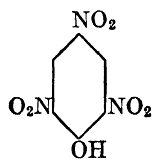
Definition of a Dye.—A dye may be defined as an organic compound containing both chromophore and auxochrome groups linked to benzene

rings. A chromophore group imparts to the compound the property of color. Compounds of benzene containing chromophore radicals have been called chromogens. Such a compound, even though it is colored, is not a dye. It possesses no affinity for, or ability to unite with, fibers and tissues. The color may be easily removed by mechanical methods. In order for a compound to be a dye it must contain not only a chromophore group but also another group that imparts to the chemical the property of electrolytic dissociation. Such groups are known as auxochromes. Auxochrome groups furnish salt-forming properties to the compound.

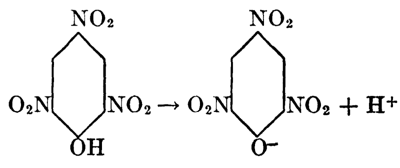
This may be illustrated by the following example: The nitro group (NO_2) may be considered a chromophore. When three hydrogen atoms in the benzene molecule are replaced by three nitro groups, the compound trinitrobenzene is formed:



This compound is yellow in color and is a chromogen but not a dye. The compound does not dissociate electrolytically and, therefore, is unable to form salts with either acids or bases. If, however, another hydrogen atom is replaced by an auxochrome group, such as (OH), the compound known as picric acid is formed:



This compound is also yellow in color and is capable of dissociating as follows:

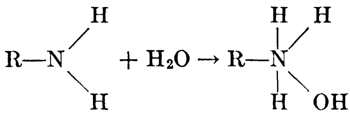


The dye portion of the molecule has a negative electrical charge. Therefore, it is an acid dye, being capable of forming salts with bases. The color of picric acid is due to the chromophore groups (NO_2), and its dyeing properties are due to the auxochromic hydroxyl group (OH), which imparts to the compound the property of electrolytic dissociation.

Acidic and Basic Dyes.—Auxochrome groups are either acidic (OH) or basic (NH_2). Acid dyes ionize to give the dye portion of the molecule or anion a negative electrical charge. Basic dyes ionize to give the dye portion of the molecule or cation a positive electrical charge.

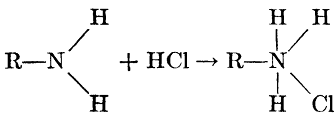
The amino group is basic by virtue of the ability of its nitrogen (N) atom to become pentavalent on the addition of water or of an acid.

With water:



Amine Organic ammonium base

With an acid:



Amine Amine hydrochloride

The hydroxyl group is acid by virtue of its power to furnish [illegible] ions by dissociation. The amino (NH_2) group is a stronger base than the hydroxyl group (OH) is an acid. If one of each of these radicals is present, the basic character of the amino radical predominates.

Some dyes have the sulfonic group (SO_2OH) attached to the benzene ring. It is a strongly acid group, possessing salt-forming properties. The radical is only weakly auxochromic. It serves two very important purposes in the dye molecule: (1) It renders insoluble dyes soluble, and (2) a basic dye is changed to one acidic in character by the introduction of the sulfonic group in the benzene ring. Since the radical is only weakly auxochromic, a compound containing a chromophore and a sulfonic acid group is not a dye unless an auxochrome radical is also present.

The dyes of commerce are not acids or bases in the true sense. Generally the basic dyes are sold as salts of colorless acids, such as acetic, hydrochloric, sulfuric. On the other hand, the acidic dyes are marketed as salts of sodium, potassium, ammonium, or calcium.

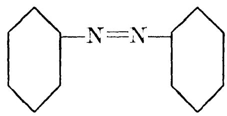
Both acidic and basic dyes are used in bacteriology. The acid dyes are used chiefly to stain cytoplasm. The basic dyes, on the other hand, stain nuclear material more intensely than cytoplasm.

Chromophores.—In order that a compound be a dye it must contain at least one chromophore group that imparts to the substance the property of color. Some of the chromophore groups are basic in character and others are acidic. The basic chromophores include (1) the azo group, (2) the azin group, and (3) the indamin group.

1. The azo group

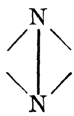
—N=N—

is found in all azo dyes. In these compounds a benzene group is attached to each atom of nitrogen. The dyes of this group may be considered as derivatives of azobenzene,



Examples of dyes containing this chromophore are methyl orange and Bismarck brown.

2. The azin group

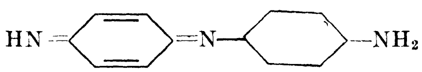


is found in the phenazins. Neutral red and the safranins are examples of azin dyes.

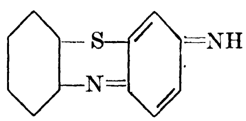
3. The indamin group

—N=

is found in the indamins, the thiazins, and others. Many of the dyes have two benzene rings attached to a nitrogen atom. One of the rings shows the quinoid structure:



The thiazins have the two benzene rings further joined together by an atom of sulfur. The simplest thiazin nucleus has the following structure:

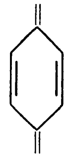


The best known dye having the thiazin base is methylene blue.

The acid chromophores include (1) the nitro group and (2) the quinoid ring.

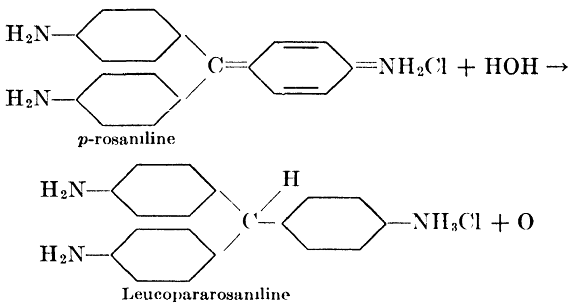
1. The nitro group (NO_2) is found in many compounds, an example of which is picric acid.

2. The quinoid ring



occurs in many dyes such as the indamins, the xanthenes, and the di- and tri-phenylmethanes. Some of the well-known dyes in this group are rosolic acid, fuchsin, the methyl violets, methyl green, crystal violet, *p*-rosaniline, etc.

The chromophores are easily reduced by combining with hydrogen at the double bonds. The nitro group may be reduced to an amino radical; the double bonds of the quinoid ring may break and one atom of hydrogen be taken up by each valence set free. A reduction of the chromophore group results in a loss of color. These decolorized dyes are known as leuco compounds. Dyes may be used as indicators of oxidation and reduction. The decolorization of *p*-rosaniline may be represented by the following equation:



CLASSIFICATION OF BIOLOGICAL DYES

The most important dyes used in bacteriology are given in Table 3. Some of them are acidic and others are basic. The basic dyes, for reasons already given, are the most important ones from the standpoint of the bacteriologist. The dyes are classified, as far as possible, according to the chromophore groups they contain.

THEORIES TO EXPLAIN STAINING

Many theories have been advanced to explain the phenomenon of staining. All of them attempt to explain the processes as purely physical or chemical phenomena.

Physical.—A physical process may be defined as a reaction between two substances in which no new compound is formed. The proponents of the physical theory claim that all staining reactions can be explained on

the basis of capillarity, osmosis, adsorption, and absorption. There appears to be no general agreement on the amount of weight that should be given to each force, although all agree that they occur in the process of staining.

Chemical.—In a chemical reaction a new compound is formed having physical and chemical properties different from the original reacting

TABLE 3.—CLASSIFICATION OF BIOLOGICAL DYES

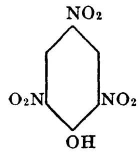
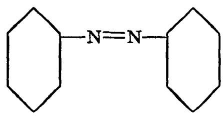
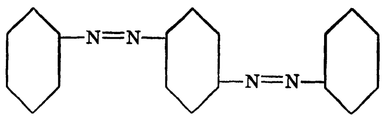
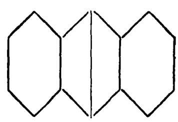
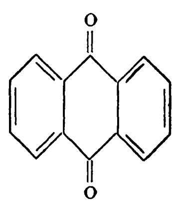
Dye	Chromophore, etc.
I. The nitro dyes 1. Picric acid 2. Aurantia 3. Martius yellow	The chromophore is —NO_2 The dyes are all acid  Picric acid
II. The azo dyes 1. Bismarck brown *Y* 2. Brilliant yellow *S* 3. Congo red 4. Janus green 5. Methyl orange 6. Methyl red	The chromophore —N=N—, joins together benzene or naphthalene rings  Sometimes the chromophore occurs more than once:  Groups in the benzene ring are usually in the para position to each other
III. The anthraquinone dyes 1. Alizarin 2. Alizarin red *S* 3. Purpurin	The dyes are derivatives of anthracene,  through the oxidized compound anthraquinone: 

TABLE 3.—(*Continued*)

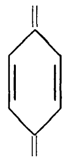
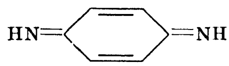
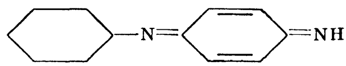
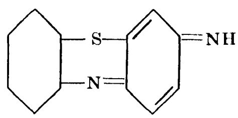
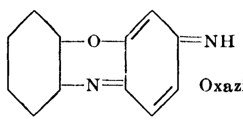
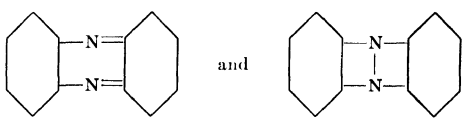
Dye	Chromophore, etc.
IV. The Quinoneimine dyes 1. The indamines No members of any importance	The dyes of this group contain two chromophores, the indamine group —N= and the quinoid ring  They are derivatives of the theoretical compound paraquinone-di-imine  In a typical formula one of the hydrogen atoms is replaced by a phenyl group: 
2. The thiazines *a.* Methylene azure *b.* Methylene blue *c.* Methylene green *d.* Methylene violet *e.* Thionine *f.* Toluidine blue *O*	The thiazines have a sulfur atom attached to both the phenyl and the quinone groups to form a third closed ring: 
3. The oxazines *a.* Cresyl violet *b.* Brilliant cresyl blue	In the oxazines, the sulfur of the thiazines is replaced by an atom of oxygen:  Oxazine
4. The azines *a.* The amidoazines (1) Neutral red (2) Neutral violet *b.* The safranines (1) Safranine *O* *c.* The indulines (1) Nigrosine	The dyes are derivatives of phenazine. Two formulas are possible  In the first formula the quinoid ring is the chromophore; in the second formula the azine group —N—|—N— is the chromophore

TABLE 3.—(*Continued*)

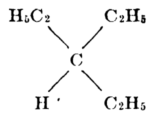
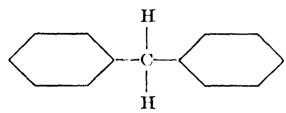
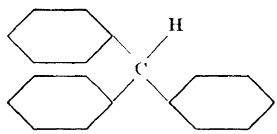
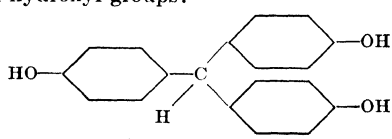
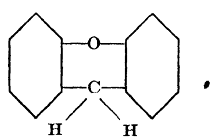
Dye	Chromophore, etc.
V. The phenylmethane dyes 1. The diamino triphenylmethane dyes *a.* Brilliant green *b.* Malachite green	This group comprises the most important dyes used in bacteriology. The compounds are substituted methanes. One or more hydrogen atoms may be replaced. If 3 hydrogen atoms are replaced by ethyl groups, the compound triethylmethane is formed:  If two hydrogens of methane are replaced by phenyl groups, diphenylmethane is formed: 
2. The triamino triphenylmethane dyes *a.* Acid fuchsin *b.* Basic fuchsin *c.* Crystal violet *d.* Ethyl violet *e.* Hofmann's violet *f.* Methyl blue *g.* Methyl green *h.* Methyl violet *i.* New fuchsin *j.* Pararosaniline *k.* Rosaniline	If three hydrogens are replaced, triphenylmethane is produced:  The introduction of amino and other groups and substituted amino groups accounts for the large number of compounds possible.
3. Hydroxy triphenylmethane dyes *a.* Rosolic acid	These are triphenylmethane derivatives in which the amino groups of the rosanilines are replaced with hydroxyl groups:  Leucorosolic acid
VI. The xanthene dyes 1. The pyronine dyes *a.* Pyronine, *B* *b.* Pyronine, *Y*	The xanthenes are derivatives of the compound xanthene: 

TABLE 3.—(*Continued*)

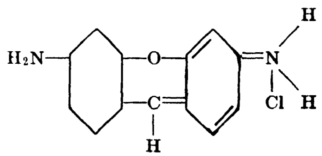
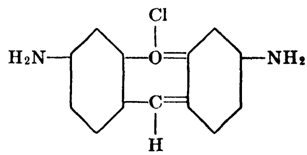
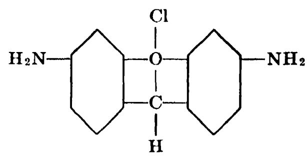
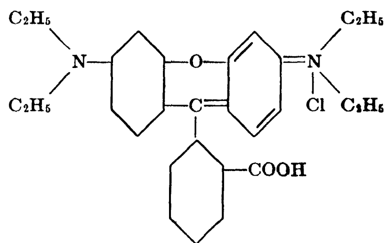
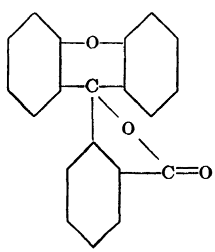
Dye	Chromophore, etc.
	The pyronines are methylated diamino derivatives of xanthene. They are closely related to the diphenylmethanes:  or  or 
2. The rhodamine dyes *a.* Rhodamine, *B*	The rhodamines are similar to the pyronines except that they contain another benzene ring with a carboxyl group in the ortho position:  Rhodamine *B*
3. The fluorane dyes *a.* Eosin, *B* *b.* Eosin, *Y* *c.* Erythrosin, *B* *d.* Erythrosin, *Y* *e.* Fluorescein *f.* Rose bengal	The fluorane dyes are derivatives of fluorane: 

TABLE 3.—(*Continued*)

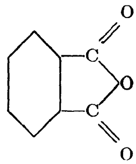
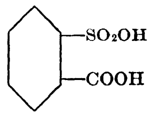
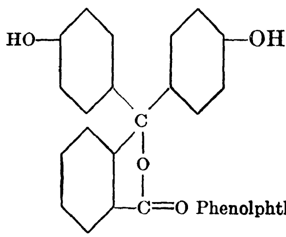
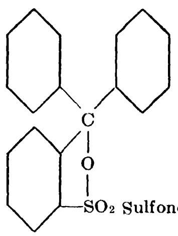
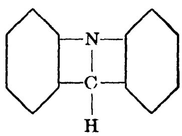
Dye	Chromophore, etc.
4. The phenolphthalein and the sulfonephthalein dyes. *a.* Bromochlorophenol red *b.* Bromocresol green *c.* Bromocresol purple *d.* Bromophenol blue *e.* Bromophenol red *f.* Bromothymol blue *g.* Chlorophenol red *h.* Cresolphthalein *i.* Cresol red *j.* Metacresol purple *k.* Phenolphthalein *l.* Phenol red *m.* Thymol blue	A phthalein is a compound of phthalic anhydride,  with phenol or a derivative of phenol. A sulfonephthalein is a compound of orthosulfobenzoic acid,  with phenol or a phenol derivative  Phenolphthalein  Sulfonephthalein
5. The acridine dyes *a.* Acriflavine *b.* Phosphine	The acridines are derived from acridine,  a compound closely related to xanthene
VII. The natural dyes. These are not so important as the artificial dyes. Also, their chemistry is not well under-	

TABLE 3.—(*Continued*)

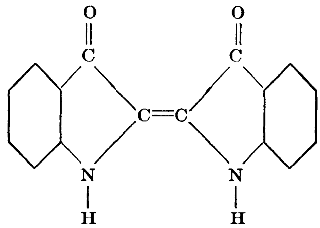
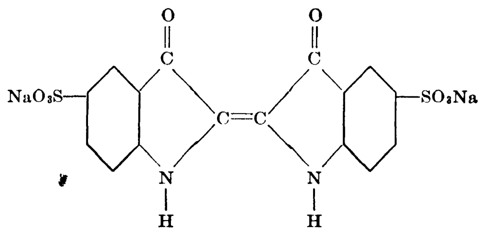
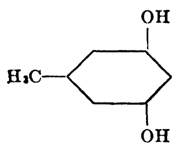
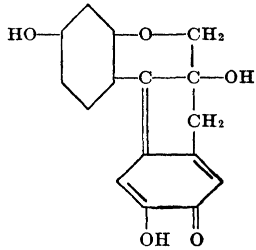
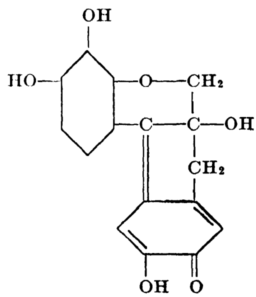
Dye	Chromophore, etc.
stood. Only a few of them are of any great importance in bacteriology. They are	
1. Indigo	Several species of plants of the genus *Indigofera* contain a glucoside, indican, which on fermentation yields the dye indigo 
2. Indigo-carmine	
3. Carmine	This dye is prepared by treating the dried bodies of the female insect, *Coccus cacti*, with water. A red dye, cochineal, is extracted which is converted into carmine by treatment with alum. The dye principle is known as carminic acid. The exact formula is not known
4. Orcein and litmus. Both dyes are obtained from the lichens *Lecanora tinctoria* and *Rocella tinctoria*	When these lichens are treated with ammonia and exposed to air, blue or violet colors develop. The colors are due to acids, one of which is known as orcin  When orcin is treated with ammonia and exposed to air, the violet dye orcein develops. The formula is not known Litmus is obtained from the same lichens as orcein. The lichens are treated with lime and potassium

TABLE 3.—(*Continued*)

Dye	Chromophore, etc.
	or sodium hydroxide. Ammonia is then added and the lichens are exposed to air for the color to develop. The formula is not known
5. Brazilin	Brazilin is obtained from the bark of brazil wood. It is colorless but on exposure to air it is oxidized to the red dye brazilein 
6. Hematoxylin	Hematoxylin is obtained from the bark of logwood by extraction. It is not a dye but, on standing in air, the hematoxylin is oxidized to the dye hematein, which is similar to brazilein in composition 

substances. Furthermore, it is impossible to recover the original reactants by means of simple solvents. When bacteria are stained, there is no evidence that the dye has been changed chemically to form a new substance. It is usually possible to extract all or nearly all of the dye from the bacterial cells by sufficiently long immersion in water, alcohol, or other solvents. The bacterial protoplasm never completely removes all of the dye from solution. This is contrary to a chemical reaction, which tends to continue until one of the components of the reaction is exhausted.

It is well established that some parts of a cell are acidic in reaction and other parts are alkaline. This fact has led chemists to explain the

phenomenon of staining on a purely chemical basis. The proponents of the theory state that the acid constituents of the cell unite or react with basic dyes and the basic constituents react with acid dyes. The nucleuses of cells that are acid in character react strongly with the basic dyes. The cytoplasm of the cell, on the other hand, is basic and reacts strongly with acid dyes. The process is not so simple as this, however, and probably does not explain all of the facts.

It is well known that bacteria possess a negative electrical charge McCalla (1940*a*,*b*) showed that bacteria attract positively charged ions, according to the equation:

$$(\text{n bases}^+) + (\text{B}^{n-}) \rightleftharpoons (\text{n bases}^+)(\text{B}^{n-})$$

where B represents the bacterial cell and *n*, an unknown number of negative ionic charges.

McCalla (1941) found that, when a negatively charged bacterial cell was treated with magnesium, the base was adsorbed to the cell until a neutral system was produced. In other words, the positively charged magnesium ions were attracted to the negative valences of the bacterial cell. If a stain, such as methylene blue, were added to the bacteria saturated with magnesium, the base was displaced by the stain, according to the equation:

$$(\tfrac{1}{2}\text{nMg}^{++})(\text{B}^{n-}) + \text{nS}^+\text{Cl}^- \rightleftharpoons (\text{nS}^+\text{B}^{n-}) + \tfrac{1}{2}\text{nMgCl}_2$$

where S represents the stain (methylene blue ion). The magnesium was displaced by the methylene blue in stoichiometrical proportions.

McCalla concluded that the reaction of stains with bacteria is an adsorption exchange process chemical in nature. Basic stains act as cations, replacing similarly charged ions from the bacterial system. These results are in support of the chemical nature of staining. Additional discussion on the chemical nature of staining is given under The Gram Stain (page 61).

Summarizing, it may be stated that more evidence is needed before it can be stated definitely that staining is physical or chemical. It is highly probable that staining is neither entirely physical nor chemical but a combination of both processes.

STAINING SOLUTIONS

Preparations employed for staining bacteria are aqueous solutions. In most cases the dyes are first dissolved in alcohol and the staining solutions prepared by diluting the alcoholic solutions with distilled water. Since alcohol removes dyes from stained cells, pure alcoholic solutions of dyes are never employed.

Staining solutions generally contain a low concentration of dye. Rarely does the dye concentration amount to more than 1 per cent. A very dilute staining solution acting for a relatively long period of time will, in general, produce much better results than a more concentrated solution acting for a short time interval. This is the method followed where it is desired to reveal the internal structure of bacteria. In actual practice, however, the more concentrated staining solutions are used because of the great saving in time. Where time is not a factor, the more dilute preparations should be employed.

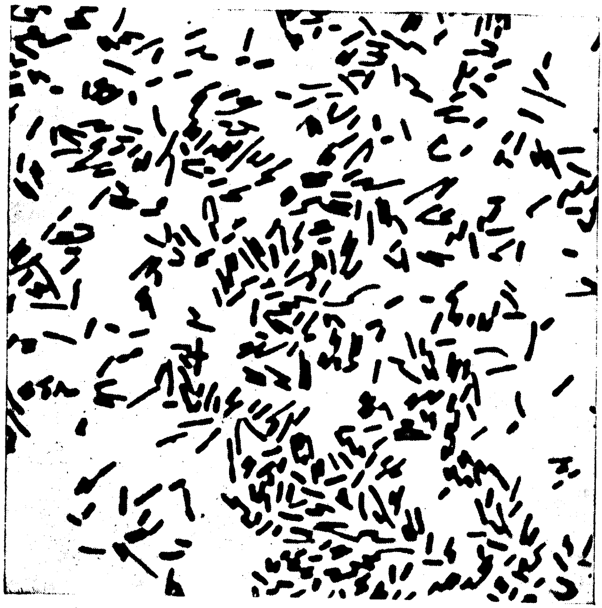


FIG. 17.—*Escherichia coli*, carbolfuchsin stain.

Intensifiers.—The addition of certain substances to staining solutions causes the organisms to take up the stain more deeply than would occur in their absence. These substances are spoken of as intensifiers. Acid dyes stain better if their solutions are made more acid; basic dyes stain better if their solutions are made more alkaline. Intensifiers include acids, alkalies, aniline oil, phenol, etc. The application of heat will, in many cases, produce the same result.

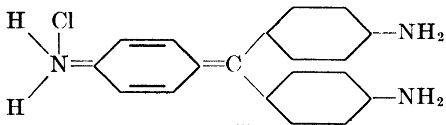
Mordants.—In the staining process substances are sometimes used that have a strong chemical affinity both for the substrate and the dye. They are chemicals that have the ability to make dyes stain objects that they would not stain otherwise. These substances are given the name of mordants. Tannic acid is an example of a mordant.

Simple Stains.—Many different kinds of staining solutions are employed in the various bacteriological procedures. Some are for general

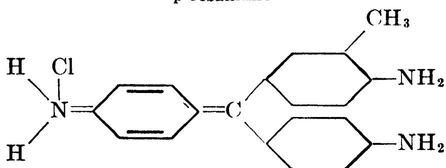
use and others are adapted to specific purposes. A simple staining solution is one that contains only a single dye dissolved in the solvent. It is applied to the bacteria in one application. The simple staining solutions that are employed probably more than any of the others for routine purposes are dilute carbolfuchsin, methylene blue, and gentian violet or crystal violet.

Carbolfuchsin Stain.—This stain is prepared by dissolving the dye basic fuchsin in a 5 per cent solution of phenol or carbolic acid. The phenol is added as an intensifier. The basic fuchsin of commerce is usually a mixture of the two dyes *p*-rosaniline and rosaniline. Sometimes new fuchsin may also be present. These compounds are members of the triamino triphenyl methane group, which comprises the most important dyes used in bacteriology.

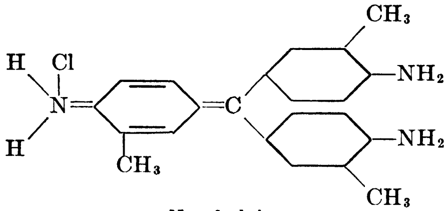
The formulas of the compounds are as follows:



p-rosaniline



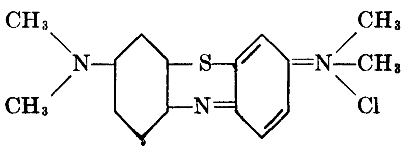
Rosaniline



New fuchsin

The more methyl groups present in the molecule the deeper will be the shade of red. New fuchsin (three methyl groups) is the deepest in shade and *p*-rosaniline (no methyl groups) the least so of the above three compounds.

Methylene Blue Stain.—Methylene blue is tetramethyl thionine, a basic ~~dye, having~~ the following formula:



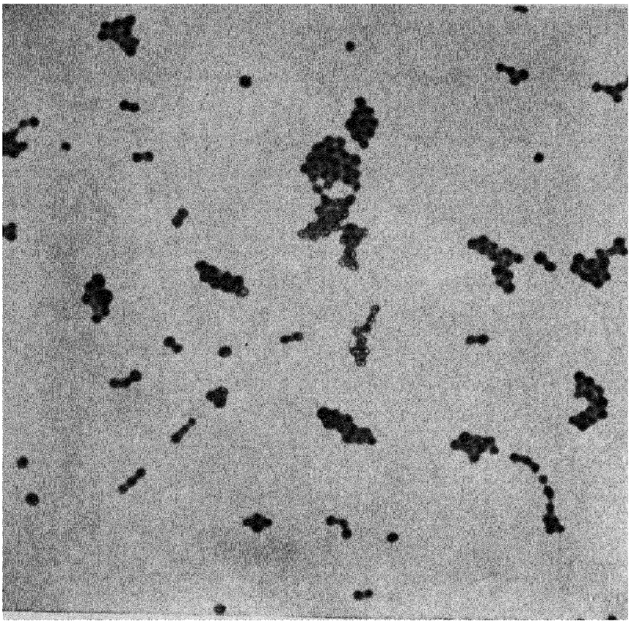


FIG. 18.—*Staphylococcus aureus*, carbolfuchsin stain.

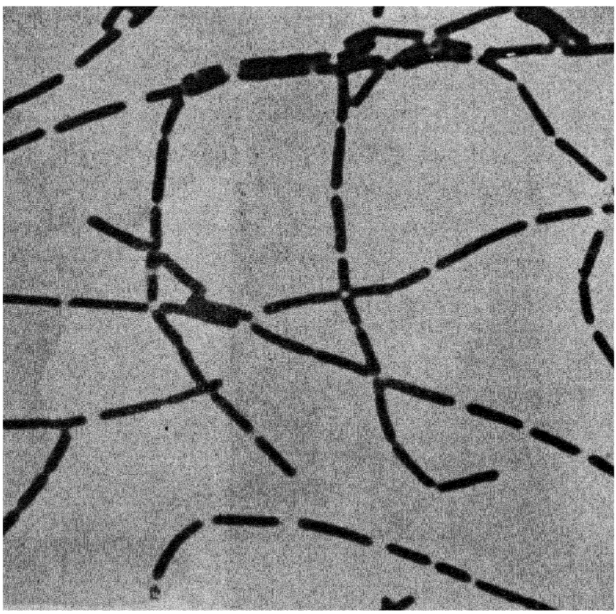
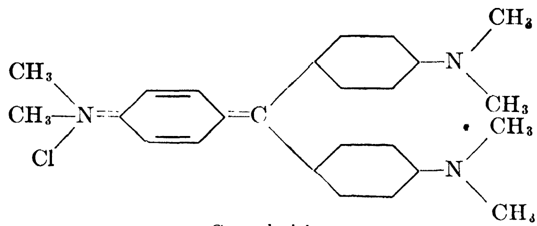


FIG. 19.—*Bacillus subtilis*, vegetative cells only. Carbolfuchsin stain.

The chromophore is the thiazine group with one of the benzene rings having a quinoid structure.

Methylene blue is a very important dye, being used more than any other stain in biological work. Because of its strongly basic nature it stains nucleuses and nucleic acid granules very intensely. It is very useful in making a rapid survey of the bacterial population of milk. This dye is usually preferred in staining smears for the diagnosis of diphtheria. It is used in combination with eosin for staining blood films. These are only a few of its many uses in bacteriology. Other uses for the dye will be taken up under the various chapters that follow.

Crystal Violet Stain.—Crystal violet is also a member of the triamino-triphenyl-methane group of compounds. Chemically it is hexamethyl *p*-rosaniline and the formula is as follows:



Crystal violet

The dye is also known as methyl violet 10*B*, gentian violet, hexamethyl violet, etc. It produces the deepest shade of the *p*-rosanilines and is considered the most satisfactory of all the violet compounds as a simple stain for general purposes.

Differential Stains.—Differential stains are composed of more than one dye. In some of the staining techniques, the dyes are applied separately; in others they are mixed and applied in one solution. The two most important differential stains used in bacteriology are the Gram stain and the acid fast stain. These will be discussed in Chap. IV, Morphology of Bacteria.

STAINING OF BACTERIA

Preparation of Smears.—A bacterial smear is prepared by removing a loopful of a liquid culture from a tube, by means of a sterile wire loop, and spreading the liquid on a glass slide over an area of about ½ sq. in. If a solid culture is used, a minute amount of the growth is first emulsified in a drop of distilled water, previously placed in the center of a glass slide, then spread out over an area of about ½ sq. in. The smear is carefully dried by holding the slide high over a low gas flame to avoid steaming. The dried smear is fixed by quickly passing the slide five or six times through the upper portion of the Bunsen flame. This prevents

the film from being washed off during the staining process. The dried and fixed smear is then covered with the staining solution and allowed to act for a definite period of time. This will vary depending upon the staining solution used. Finally the slide is washed in water, dried by blotting, and examined under the microscope.

Viability of Fixed and Stained Organisms.—It is generally stated that bacteria in dried, fixed, and stained smears are no longer viable and that no danger from infections is possible if pathogenic organisms are so treated. Thurn (1914) reported that organisms are not necessarily killed in fixed and stained preparations. He found that cultures of *Staphylococcus aureus*, *Eberthella typhosa*, *Escherichia coli*, *Bacillus anthracis*, *Vibrio comma*, *Corynebacterium diphtheriae*, and *Saccharomyces cerevisiae* smeared onto glass slides and fixed by passing three times through the flame, but not stained, still contained viable organisms. Eighteen preparations of pathogenic and nonpathogenic organisms failed to show viable organisms after staining by the Gram technique. On the other hand, *B. anthracis* survived 1 min. and *B. mesentericus* 3 min. of treatment with carbolfuchsin and both organisms survived 5 min. of treatment with methylene blue. More recently Morton (1939) also showed that organisms are able to survive drying and fixation by heat. In addition he reported that certain organisms are capable of surviving treatment with basic fuchsin, Hucker's gentian violet, aqueous safranine, and methylene blue stains.

More care should be used in the handling of stained preparations in the laboratory, especially if pathogenic organisms are employed, since the process of staining is no indication that the organisms are necessarily killed.

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CHAPTER IV

MORPHOLOGY OF BACTERIA

GENERAL CONSIDERATIONS

Bacteria belong to the great class of organisms known as the Schizomycetes (*schizo-*, fission, and *mycetes*, fungi). The organisms grouped in this class are so named because they reproduce typically by cell division or fission.

The class Schizomycetes is divided into seven orders. One of these orders, the Eubacteriales, includes all of the common or true bacteria. The other six orders embrace those organisms possessing characteristics intermediate between the true bacteria and other plants or animals.

Bacteria are characterized as typically unicellular plants, the cells being usually small and relatively primitive in organization. The cells may be spherical, cylindrical, spiral, or filamentous, and are often united into chains or into flat or cubical aggregates. The filamentous cells may show false, or more frequently, true branching. Multiplication occurs normally by cell division. Endospores are formed by some species of the Eubacteriales while conidia are produced by some of the filamentous forms. Certain of the sulfur bacteria produce a red-colored pigment known as bacteriopurpurin, which appears to function somewhat in the manner to chlorophyll in higher plants, when the bacteria are exposed to the light. Many species produce pigments of other types. The cells may be motile by means of long, whip-like processes known as flagella (singular, flagellum). Some of the forms intergrading with the protozoa are flexuous. A few of the higher filamentous bacteria show an oscillatory movement similar to that observed in certain of the blue-green algae.

Shape of Bacteria.—Bacterial cells exhibit three fundamental shapes: the spherical, the rod, and the spiral forms. All bacteria exhibit pleomorphism in more or less degree, under normal or other conditions, but a bacterial species is still generally associated with a definite cell form when grown on standard media under certain specified conditions.

Some of the round or coccus forms (singular, coccus; plural, cocci) are apparently perfect spheres; others are slightly elongated or ellipsoidal in shape. Spherical forms that grow normally in pairs (diplococci), fours (tetrads), or chains (streptococci) are usually slightly flattened at their adjacent surfaces. A pair of such organisms is usually referred to as coffee-bean shaped.

The rod forms also show considerable variation. A rod is usually considered to be a cylinder with the ends more or less rounded. Some rod forms are definitely ellipsoidal in shape. The ends of rods also show considerable variation. Some species are markedly rounded and others exhibit flat ends perpendicular to the sides. Gradations between these two forms may be seen.

Rods may show marked variation in their $\frac{\text{length}}{\text{width}}$ ratio. Some rods are very long in comparison to their width and others are so short they may be confused with the coccus forms.

The shape of an organism may also vary depending upon certain environmental factors, such as temperature of incubation, age of the culture, concentration of the substrate, and composition of the medium. Bacteria exhibit their characteristic morphology usually in young cultures and on media possessing favorable conditions for growth. Henrici (1928) distinguishes three forms of the bacteria: (1) the embryonic form, (2) the mature form, and (3) the senescent form. The embryonic forms correspond to the growth phase and are characterized by long, slender, and uniform cells. The mature forms correspond to the resting phase and are characterized by cells shorter in length and more variable in form, but less so in size. The senescent forms correspond to the death phase and are characterized by great variability in both form and size.

Those forms which depart widely from the standard morphological picture, when one or more environmental factors are changed, have been called involution forms and forms of degeneration. Henrici prefers to call them senescent forms because he believes that an organism changes morphologically as it becomes older just as a multicellular organism changes with age. Some morphologists have considered them as definite stages in an orderly life cycle of an organism.

Many experiments may be cited showing the effect of environmental changes on the growth of bacteria. It is well known that *E. coli*, when inoculated into a medium having a low surface tension (sodium ricinoleate broth), grows in the form of long filaments whereas the same organism, inoculated into a medium having a high surface tension (calcium chloride broth), grows as very short rods almost spherical in form. Metchnikoff (1888) inoculated the organism causing tuberculosis into a medium containing 12 per cent of glycerin and incubated the culture at 40°C. He reported extensive branching of the organisms whereas such forms are very rarely, if ever, encountered on the usual laboratory media incubated at 37°C.

Bacterial variation resulting from changes in age and other environmental factors are only temporary. The original forms reappear when the organisms are inoculated into fresh medium.

Size of Bacteria.—Bacteria are considerably smaller than yeasts, molds, algae, and protozoa. They vary greatly in size according to the species. Some bacteria are so small that they cannot be easily seen with a powerful microscope. Regardless of their size none are visible with the naked eye.

A spherical or coccus form is measured by the size of its diameter; a rod or spiral form by its length and width. Calculation of the length of a spiral organism by this method gives only the apparent length and not the true length. The true length may be computed by actually measuring the length of each turn of the spiral. Mathematical expressions have been formulated for making such computations.

The method employed for fixing and staining bacteria may make a difference in their size. The bacterial cell shrinks considerably during drying and fixing. This will vary somewhat depending upon the type of medium employed for the cultivation of the organisms. The magnitude of the shrinkage will average about one-third of the length of the cell when compared to an unstained, hanging-drop preparation. Knaysi (1938) reported a shrinkage of from 15 to 25 per cent when young cells of *Bacillus megatherium* were transferred from nutrient broth to a similar medium containing sodium chloride in two-molar concentration. The usual laboratory media contain sodium chloride in about a $\frac{1}{10}$-molar concentration.

The measurements will show some variation depending upon the staining solution and the mode of application. In dried and fixed smears the cell wall and slime layer do not stain with weakly staining dyes such as methylene blue but do stain with such intensely staining dyes as *p*-rosaniline, new fuchsin, crystal violet, and methyl violet. The cell wall and slime layer are included in negatively stained preparations but the organisms measure larger than their true size owing to the fact that the colloidal dye film retracts on drying. In the living condition the cell wall and the slime layer cannot be seen and measurements of such organisms include only the cytoplasm.

All organisms that have been studied and classified have been measured. The measurements have been carried out for the most part on fixed and stained preparations. In some instances dried, negatively stained smears were used and in a few cases living material was employed. It follows from this that the method employed should be specified when measurements of bacteria are reported. Unless this is done, the figures will not have very much significance for reasons already given.

The unit of measurement is the micron, expressed by the symbol μ. It is 0.001 mm. or 0.0001 cm. A millimicron is 0.001μ or 0.000001 mm. It is expressed by the symbol $\mu\mu$.

Bacteria show considerable variation in size. Some measure as large as 80μ in length and others are as small as 0.1μ. The large forms are members of the sulfur and iron bacteria, which show characteristics intermediate between the true bacteria and higher plants. However, the majority of organisms, including the pathogenic bacteria, are about 0.5μ in diameter for the cocci and 0.5 by 2 to 3μ for the rod forms. Organisms producing spores are, as a rule, larger than the nonspore-bearing rods. Some measurements on the typical or true bacteria (Eubacteriales) are as follows: *Escherichia coli*, 0.5 by 1.0 to 3.0μ; *Eberthella typhosa*, 0.6 to 0.7 by 2.0 to 3.0μ; *Streptococcus lactis*, 0.5 to 1.0μ in diameter; *S. pyogenes*, 0.6 to 1.0μ in diameter; *Lactobacillus acidophilus*, 0.6 to 0.9 by 1.5 to 6.0μ; *Staphylococcus aureus*, 0.8 to 1.0μ in diameter; *Bacillus subtilis* rods, 0.8 by 1.5μ; *B. megatherium* rods, 1.0 to 1.5 by 3.0 to 6.0μ; and *B. anthracis* rods, 1.0 to 1.5 by 5.0 to 10.0μ.

The most commonly employed method for measuring bacteria is by means of the ocular micrometer. Measurements may also be made by using a camera lucida attachment and drawing oculars, or by projecting the real image on a screen and making the measurements.

The same factors that cause variations in the shape of bacteria also produce alterations in their size. With one or two exceptions young cells are much larger than older or mature cells. Knaysi (1938) showed that cells of *B. subtilis* from a 4-hr. culture may be from 5 to 7 times longer than cells from a 24-hr. culture. Variations in width are much less pronounced. A notable exception to the rule of decreasing cell size with age is the causative organism of diphtheria (*Corynebacterium diphtheriae*).

A decrease in cell length and width appears to be due to a variety of factors. Changes in the environment with an accumulation of toxic

TABLE 4.—INTERRELATION OF PARTICLE SIZE OF BACTERIA, VIRUSES, MOLECULES, ATOMS, RADIATIONS, AND BASIC UNITS

Up to 10^{-4} cm. cells	10^{-4} to 10^{-6} cm. viruses	10^{-6} to 10^{-8} cm. molecules	10^{-8} to 10^{-9} cm. atoms	10^{-10} cm. radiations	10^{-11} to 10^{-13} cm. basic units
Staphylococcus 1×10^{-4}	Psittacosis 3×10^{-5}	Proteins $4\text{–}9 \times 10^{-7}$	Diameter of H atom 1×10^{-8}	Gamma rays from radium	Neutron
	Variola 2×10^{-5}	Small colloid particles	X rays		Electron
	Herpes 1.2×10^{-5}				Cosmic rays
	Poliomyelitis 1×10^{-6}				Alpha rays
					Proton

waste products in the medium appear to be the major causes. Also, an increase in the osmotic pressure of the medium will cause a decrease in cell size and may possibly be the most important factor.

The interrelation of particle size of bacterial cells, viruses, molecules, atoms, radiations, and basic units, according to Zangger, is given in Table 4.

Presence of a Nucleus.—Bacteria, as a group, are not all alike in their morphological picture. Differences in structure do exist between species. They display a marked degree of morphological differentiation which may be associated with a highly complex life cycle. It is generally agreed that a bacterial cell consists of a compound membrane enclosing cytoplasm and often cytoplasmic inclusion bodies, and a nucleus. The term protoplasm is commonly used to denote both cytoplasm and nucleus. In addition, some species contain resistant bodies known as spores, and some are surrounded by organs of locomotion called flagella.

There is still considerable doubt as to the presence of a well-defined nucleus in typical bacterial cells. Nuclear studies have been concerned mainly with the organisms classified with the higher bacteria having characteristics intermediate between the true bacteria and higher plants or animals. The organisms studied are for the most part very large. It is doubtful if any worker has conclusively demonstrated the presence of a well-defined nucleus in an organism belonging to the order Eubacteriales, or true bacteria.

Some of the earlier cytologists maintained that bacteria were very primitive forms devoid of nucleuses and consisting simply of cytoplasm, granules, and vacuoles. Others held the view that the whole bacterial body should be regarded as a "naked nucleus" corresponding to the nucleus of higher organisms. Those opposing this theory have advanced experimental evidence to show that neither cytoplasm nor nucleus can exist independently for any length of time. They oppose the use of the term "naked nucleus."

In the great majority of species of bacteria nothing can be demonstrated other than chromatin granules scattered throughout the cytoplasmic material. The chromatin is identified by its behavior either toward dyes or to other physical and chemical tests. Considerable confusion is caused by the fact that a reserve food material known as volutin (metachromatin) is chemically very similar to chromatin and gives similar reactions.

For information in favor of the presence of a definite nucleus in a species of *Staphylococcus* see the report by Knaysi (1942).

Cell Inclusions in Bacteria.—Cell-sap vacuoles have been identified in young bacteria. As the cells approach maturity, some of the water-soluble reserve food materials manufactured by the cell dissolve in the

vacuoles. Insoluble constituents precipitate out as cytoplasmic inclusion bodies.

The best-known inclusion bodies identified in bacterial cells are known as volutin. These are also called metachromatic granules, sporogenic granules, polar granules, Neisser's bodies, and Babes-Ernst bodies (Fig. 20). Such granules have been confused with chromatin but they do not exhibit the characters of nuclear material. Nucleuses stain very intense with basic coal-tar dyes. Metachromatic granules also take a deep stain with basic dyes. Chromatin contains nucleic acid. Metachromatic granules are believed to be composed of nucleic acid combined

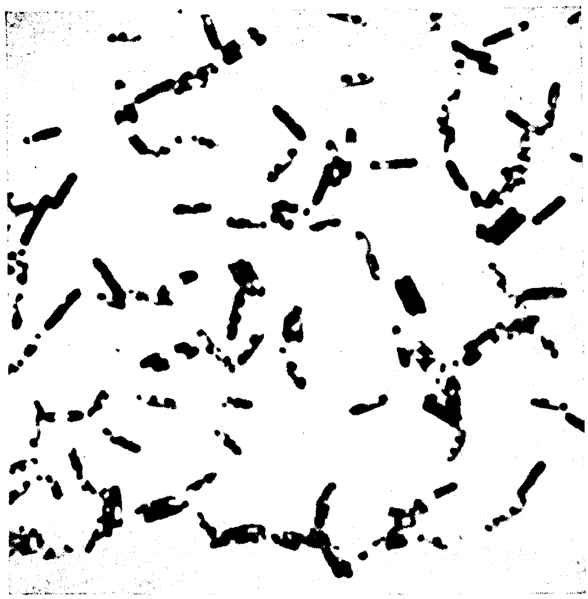


FIG. 20.—Metachromatic granules in *Bacillus subtilis*. Culture 18 hr. old and stained with a 1:5000 solution of crystal violet.

with an organic base. Chemically nucleuses and metachromatic granules are very similar.

Metachromatic granules are believed not to be living constituents of either cytoplasm or nucleus but merely stored-up reserve food material of a nitrogenous nature. Usually metachromatic granules are not seen in young cells, *i.e.*, organisms in the active growth phase. They are formed in old cells after growth has ceased. As a general rule, young cells stain solid with the usual laboratory stains and old cells display a very granular appearance.

Cell Membrane.—According to Knaysi (1938) the bacterial cell is surrounded by three membranes: (1) the cytoplasmic membrane, (2) the cell wall, and (3) the slime layer.

The cytoplasmic membrane first appears in young cells as an interfacial fluid film, becoming thicker and denser as surface active material accumulates. It is finally converted into a firm structure composed sometimes of several layers. Knaysi believes it is composed of lipoids and lipoproteins. It is the membrane principally responsible for the Gram and acid-fast reactions. When a cell is plasmolyzed by immersion in a hypertonic solution, this membrane is drawn in with the cytoplasmic material.

The cell wall is a more rigid structure and is responsible for the form of the bacterial body. It behaves as a semipermeable membrane and apparently plays a fundamental role in the life activities of the cell. The cell wall has a low affinity for dyes. This means that it is probably not stained by the usual procedures followed in staining bacterial smears. Chemically it appears to be composed of a complex carbohydrate of unknown nature and usually referred to as a hemicellulose. This carbohydrate is believed to be impregnated with other substances, some of which contain nitrogen. A polymerized, acetylated glucosamine known as chitin has been reported to be present in the walls of bacteria. This compound has been identified in the cell walls of molds.

The slime layer is considered to be a modified outer layer of the cell wall. The two structures give, in many instances, the same microchemical tests. The soluble carbohydrates elaborated by many bacteria, such as the various types of pneumococci and certain streptococci for example, have their origin in the slime layer. The slime layer, like the cell wall, has also a low affinity for dyes. When the slime layer is large and remains fixed around the cell, it is termed a capsule.

Capsules of Bacteria.—Capsules are mucilaginous or gummy envelopes of a carbohydrate nature. They are formed from the external or slime layer of bacteria. Some believe that all organisms produce small amounts of capsular material. A few species are surrounded by relatively large capsules, which can be readily seen by appropriate staining methods and their presence may be used for diagnostic purposes (Fig. 21). As a rule, an organism capable of producing a relatively large capsule does not do so under all cultural conditions. Certain conditions must be satisfied in culture media for good capsule formation.

Hoogerheide (1939) showed that the encapsulation of *Klebsiella pneumoniae* (Friedländer's bacterium) occurred when conditions were unfavorable for growth and not when active multiplication was taking place. Freshly isolated mucoid strains of the organism should not be transferred to fresh medium at short intervals but should be stored as long as possible before making new cultures. A medium low in nutrients is more favorable to capsule formation than one containing an abundance of nutrient substances.

In a later communication Hoogerheide (1940) showed that inhibition of capsulation of *K. pneumoniae* can be brought about by a great variety of electrolytes. The adsorption of electrolytes on surfaces, in general, follows the lyotropic series and increases in the direction Li < Na < K, etc. Hoogerheide concluded that, inasmuch as the inhibition of encapsulation also follows these lyotropic series, it might be possible that physical adsorption of an electrolyte on the bacterial cell, and perhaps on the enzymatic systems which are responsible for polysaccharide synthesis, is sufficient to inhibit these enzymes.

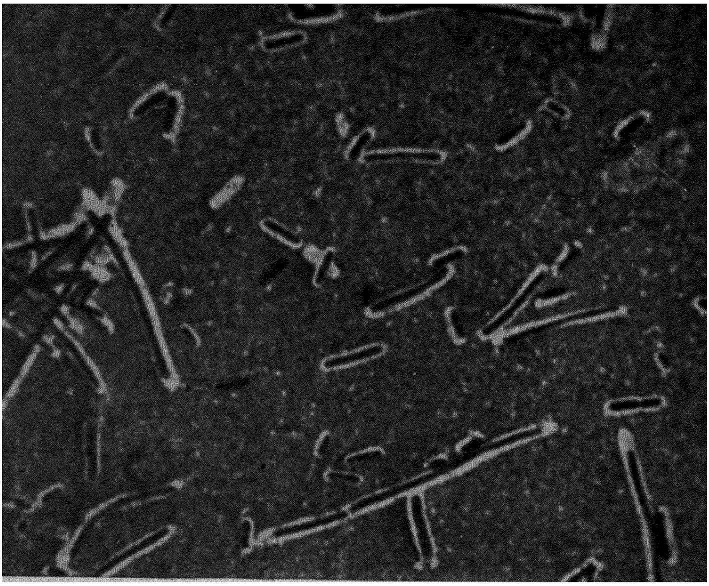


FIG. 21.—*Salmonella schottmuelleri*, mucoid type. Capsule stain, Gins' method. (*Slide prepared by H. R. Morgan.*)

Capsules appear to be developed more strongly among the pathogenic organisms. It is not a degenerative process, as was formerly supposed by some workers, but an active bacterial reaction accompanied by an increase of virulence and resistance to immune sera and to phagocytosis. The capsule functions as a protective mechanism to the bacterial body when threatened by the defensive mechanisms of the host.

Chemical analyses of capsular material recovered from several bacterial species have shown them to be complex carbohydrates known as polysaccharides. They are sometimes referred to as carbohydrate gums.

Polysaccharides that are morphologically evident as well-defined capsules are difficult to distinguish from those gums which diffuse away from the cells as they are formed. Gums of the latter type are not con-

sidered to be true cellular constituents. Organisms producing such gums do so when grown in certain sugar solutions. Some organisms produce gums only in the presence of a specific sugar; others produce them in the presence of any one of several sugars. In the absence of sugar very little, if any, gum is formed. Organisms producing gums of this type are the cause of considerable loss in the sugar industry. The increased viscosity, due to the presence of the gums, interferes with the filtration of the sugar solution.

Gums are classified as levulans, arabans, dextrans, galactans, etc., depending upon the sugars produced when the polysaccharides are hydrolyzed. Some organisms produce gums that yield only one sugar on hydrolysis and others give more than one kind of sugar. The names of a few organisms producing soluble gums and their products of hydrolysis are given in Table 5.

TABLE 5

Organism	Gum	Products of hydrolysis
Leuconostoc mesenteroides	Dextran	Glucose
Azotobacter chroococcum	Araban	Arabinose
Bacillus subtilis	Levulan	Levulose
B. mesentericus	Levulan	Levulose
Diplococcus pneumoniae, Type I	Soluble specific substance	Amino sugar, galacturonic acid
D. pneumoniae, Type II	Soluble specific substance	Glucose, aldobionic acid (glucose + glycuronic acid)
D. pneumoniae, Type III	Soluble specific substance	Glycuronic acid, galactose
D. pneumoniae, Type IV	Soluble specific substance	Acetic acid, amino sugar, glucose
D. pneumoniae, Type VIII	Soluble specific substance	Glucose, aldobionic acid
D. pneumoniae, Type XIV	Soluble specific substance	Glucosamine, galactose, acetic acid

Motility of Bacteria.—Bacterial motion is due to the presence of organs of locomotion known as flagella (singular, flagellum). They were first observed in stained specimens by Cohn (1875). He succeeded in staining them with logwood extract (haematoxylin) and subsequent treatment with chromic acid. A few of the motile sulfur bacteria are exceptions in that they do not possess flagella but exhibit a slow, creeping motion caused probably by a contraction of their protoplasm. The presence of flagella does not mean that the organisms are always motile but indicates a potential power to move.

Independent bacterial motion is a true movement of translation and must be distinguished from the quivering or back-and-forth motion

exhibited by very small particles suspended in a liquid. This latter type of motion is named Brownian movement after Robert Brown (1828, 1829, 1830) who was the first to record such an observation. It is due to the bombardment of the very small particles by the molecules of the suspending fluid.

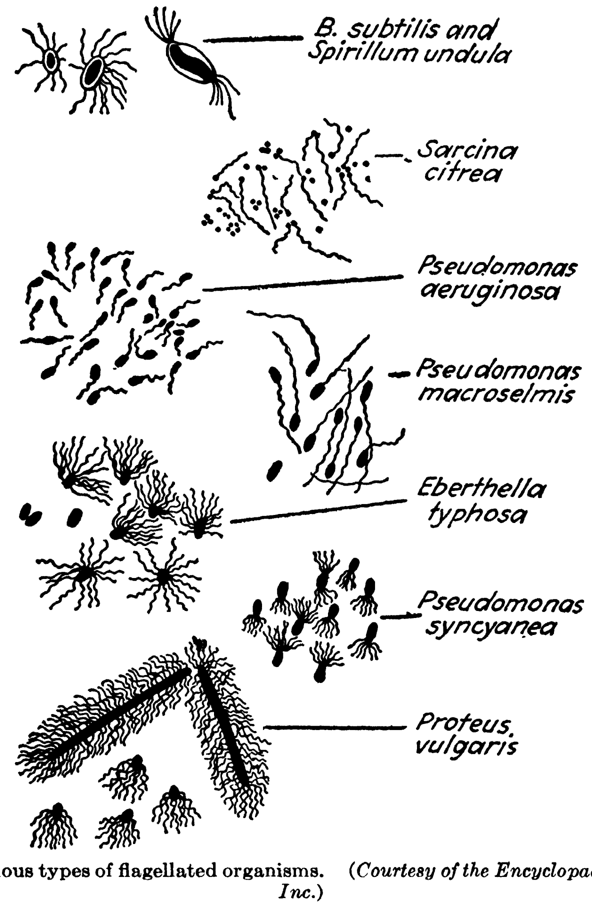


Fig. 22.—Various types of flagellated organisms. (*Courtesy of the Encyclopaedia Britannica, Inc.*)

Flagella are very delicat... and easily destroyed in the usual method of preparing smears. In the stained state they are long, slender, undulating organs with ends in some cases blunt and in others slightly thickened. The flagella are always directed backward to the direction of motion of the cell at an angle of about 45 deg. Reversal of direction takes place by swinging the flagella through an angle of about 90 deg. Turning movements take place by swinging the flagella forward on one side only. They propel the organism by means of a spiral or a corkscrew motion.

The length of flagella shows considerable variation, depending to a large extent upon age and changes in the environment. There appears to be a certain inverse relation between the length of flagella and that of the organism. Usually the shorter the cell the longer will be the flagella.

The number and arrangement of flagella vary with different bacteria, but they are generally constant for each individual species. Some have only one flagellum: others have two or more. Also the arrangement about the organisms varies considerably. Therefore, presence, number,

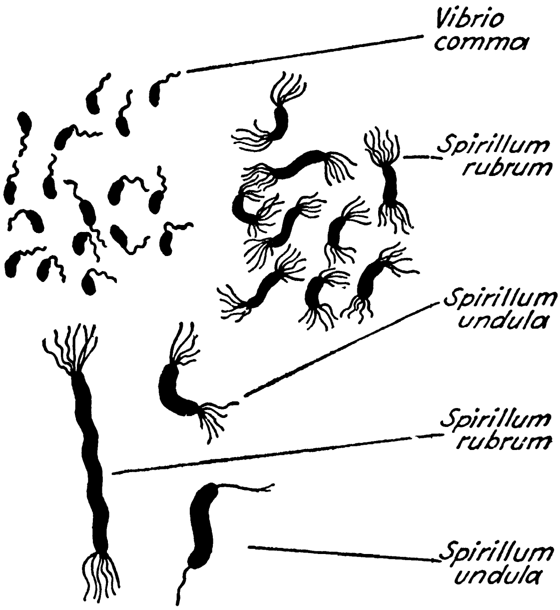


FIG. 23.—Various types of flagellated organisms. (*Courtesy of the Encyclopaedia Britannica, Inc.*)

and arrangement of flagella are used for identifying and classifying organisms (Figs. 22 and 23).

Organisms may be classified as follows on the basis of the number and arrangement of flagella:

Monotrichous—a single flagellum at one end of the cell.
Lophotrichous—two or more flagella at one end or both ends of the cell.
Amphitrichous—one flagellum at each pole.
Peritrichous—flagella surrounding the cell.

The staining of flagella is not a simple technique. For this reason many different methods have been proposed. There is little evidence to show that one method is better than any other. Therefore, the important thing to do is to select one method and familiarize yourself thoroughly with it until good results are obtained. Some bacteria may stain better

by one method than by another but there is no evidence to support this view.

Recently Conn and Wolfe (1938) gave the following precautions to be observed for preparing good flagella stains:

Young, vigorous cultures are absolutely necessary for successful flagella preparations. Agar cultures, from 18 to 22 hr. old, appear to give the best results. Old stock cultures should be transferred daily for several days to restore their vigor.

All slides must be scrupulously clean. They should be boiled in dilute cleaning solution (potassium dichromate dissolved in sulfuric acid)

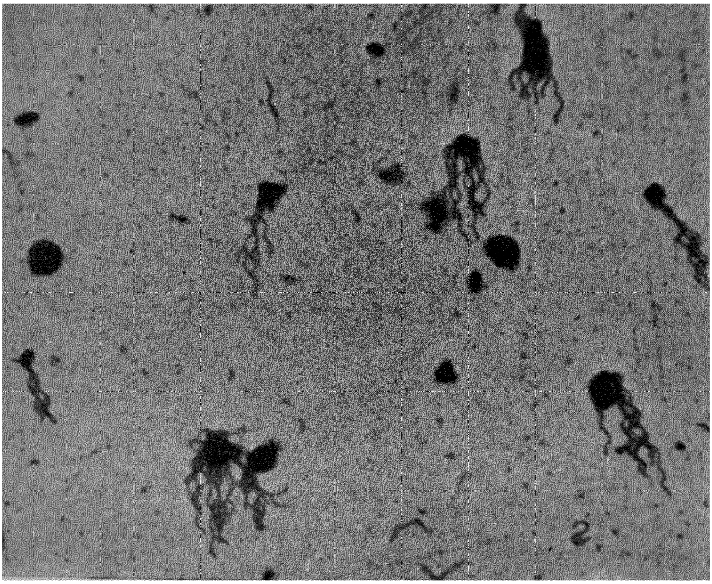


FIG. 24.—*Proteus vulgaris*, flagella stain.

for several minutes. The slides are then thoroughly rinsed in tap water followed by immersion in alcohol. Always hold the slides by the edges to avoid redeposition of grease on their surfaces. When ready to use, the slides are removed from the alcohol and burned in the flame. They are then placed on a wire gauze and strongly heated with the Bunsen burner to remove the last traces of grease.

A small amount of growth is emulsified in a tube of sterile distilled water (5 to 10 cc.). The water must have the same temperature as that of the laboratory. Poor preparations usually result from suspensions made in water that is too hot or too cold.

The suspension is allowed to stand for from 5 to 30 min. depending upon the type of organism to be stained. Gum-producing organisms

require 30 min. whereas nongum-producing organisms require only 5 to 10 min. Standing in water should be just long enough to allow the flagella to become untangled. If the bacteria remain in the water too long, the flagella break off.

A loopful of the suspension near the surface is removed and placed on a prepared glass slide. The motile cells are found near the surface whereas the nonmotile cells settled out. The loopful is smeared out thinly by touching it with the edge of a second slide and drawing it over the surface by the same method used in preparing a blood film. Rapid drying is necessary to minimize distortion.

The slide is now ready to be stained. Regardless of which method is followed the film must be treated first with a mordant to make the flagella take the stain. After the mordant has been applied to the smear, the flagella are easily stained by an appropriate dye.

Motion of Colonies.—In 1904 Muto described an organism (*Bacillus helixoides*) that exhibited colonial motility. He appears to be the first worker to record such an observation. Later Smith and Clark (1938) and Shinn (1938) reported a similar phenomenon by an organism named *B. alvei*. Since the published descriptions of the two organisms are very similar, it may be that they are different names for the same species.

Shinn prepared lapse-time motion pictures of the individual colonies on agar plates and was able to measure their velocities. He reported that the linear motions of colonies measuring 0.2 to 0.5 mm. in diameter averaged about 14 mm. per hour. Comparing this figure with the speed of individual cells of other species of motile bacteria, he obtained the following results:

Eberthella typhosa	65 mm. per hr.
Bacillus megatherium	27 mm. per hr.
B. alvei (colonies)	14 mm per hr.

The colonies exhibited not only linear motion but also a slow rotary movement. The direction of rotation of 200 to 300 colonies observed was counterclockwise, with the exception of two colonies that moved clockwise.

It is a difficult matter to explain the motility of entire colonies of bacteria. It is true that the individual cells are motile but that does not explain the unified action of the cells so that the colony moves as a whole rather than spreading in all directions as is the case with other highly motile species of bacteria.

Spores of Bacteria.– Spores are resistant bodies produced within the cells of a considerable number of bacterial species.

Spores are more resistant to unfavorable environmental conditions, such as, heat, cold, desiccation, osmosis, and chemicals, than the vegeta-

tive cells producing them. They are believed to be produced when the environment becomes too unfavorable for the existence of the vegetative forms. As far as is known, all spores produced by the true bacteria are nonmotile.

Apparently the first sign preceding the appearance of spores is the cessation of growth. The cytoplasm of the cell becomes vacuolated and filled with one or more granules. The granules gradually enlarge and at the same time increase in refractility. As the spore develops, refractility increases. As refractility increases, permeability of the spore membrane

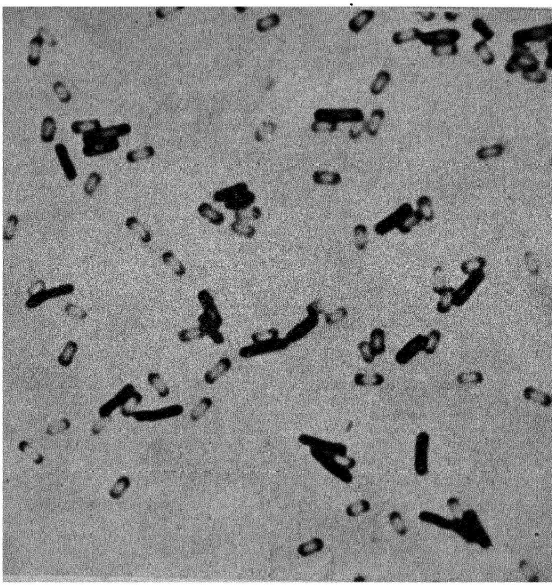


FIG. 25.—Spores and bacilli of *Bacillus subtilis*. Culture 24 hr. old.

to dyes decreases. The membrane of a fully developed spore is only faintly stained by the usual staining solutions (Fig. 25).

As a general rule, each species of spore-bearing bacillus has normally its own characteristic size, shape, and position for the spore, but this is subject to considerable variation under different environmental conditions. Bayne-Jones and Petrilli (1933) and others have given figures to indicate the degree of variation of spore size in *Bacillus megatherium*. It has already been shown that the vegetative cells producing the spores may also show considerable variation in size. The shape of spores of *B. megatherium* is usually described as being ovoid with squared ends, but it has been shown that spherical and bean-shaped forms may also be present in the same culture. The position of the spore in the cell may be central, subterminal, or terminal, but here again variation

may be shown to exist in a culture of the same organism. Each cell usually produces only one spore but occasionally two spores may be present. It may be concluded that, although the size, shape, position, and number of spores in a cell are usually quite constant, variations do exist under changing environmental conditions.

A fully grown spore may possess a diameter greater than that of the vegetative cell. The resulting forms are known as clostridium, if central, and plectridium, if terminal. Such spores are common among the anaerobic bacteria. As the spore becomes fully ripened, the sporangium gradually degenerates and finally disappears, leaving the spore in a naked state.

Very little is known concerning the composition of spores. It is usually stated that the great resistance exhibited by spores to adverse environmental changes is due to their low water content. It is known that the temperature required to coagulate protoplasm increases as the water content decreases. Recently Henry and Friedman (1937) and Friedman and Henry (1938) have shown that spores have about the same moisture content as the vegetative cells producing them. However, the spores were found to have a far greater water-binding capacity than did the vegetative cells. These investigators advanced the theory that the greater heat resistance of bacterial spores is due in part at least to the relatively high percentage of water in the bound state. Spores contain a high lipoid content, which is probably largely responsible for their increased impermeability to dyes and other substances.

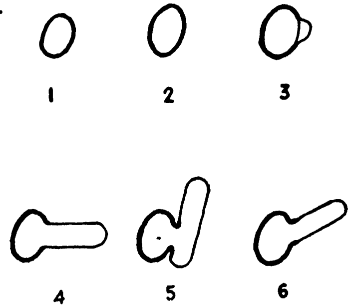


FIG. 26.—Equatorial germination without splitting along transverse axis. (*After Lamanna.*)

When a spore is removed from an unfavorable environment and placed in a suitable medium, germination occurs. Spores germinate in a variety of ways. There is a considerable degree of constancy in the method of germination of spores of the same species. Lamanna (1940) classified the modes of germination as follows:

I. Spore germination by shedding of spore coat. Characteristics of this method are:
   A. Spore does not expand greatly in volume previous to the germ cell breaking through the spore coat. The limit of volume increase of the spore may be considered to be twice its original volume.
   B. Spore coat does not lose all of its refractive property previous to germination.
   C. After the second division of the germ cell, giving a chain of three organisms, the original spore coat, remaining attached to the cells, is visible for a long time after germination.
      1. Equatorial germination (Figs. 26 and 27).

2. Polar germination (Fig. 28).
3. Comma-shaped expansion (Fig. 29).

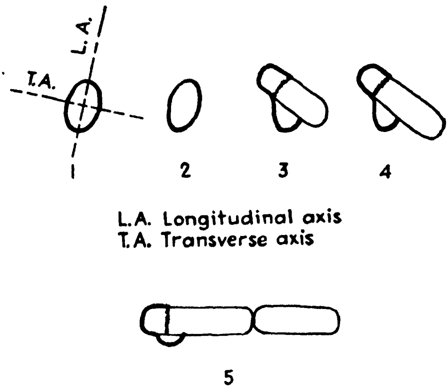


Fig. 27.—Equatorial germination with splitting along transverse axis. (*After Lamanna.*)

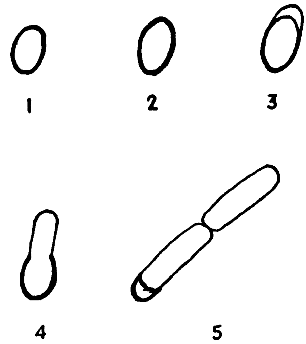


Fig. 28.—Polar germination. (*After Lamanna.*)

II. Spore germination by absorption of the spore coat. Characteristics of this method are:
   A. The spore expands greatly during germination. A tripling or greater increase of the original volume occurs (Fig. 30).
   B. The spore loses its characteristic refractiveness during germination so that it is difficult to say when the spore has disappeared and the germ cell appeared.

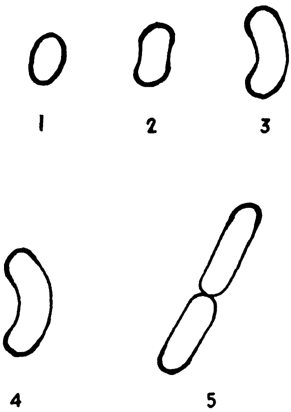


Fig. 29.—Spore germination by comma-shaped expansion. (*After Lamanna.*)

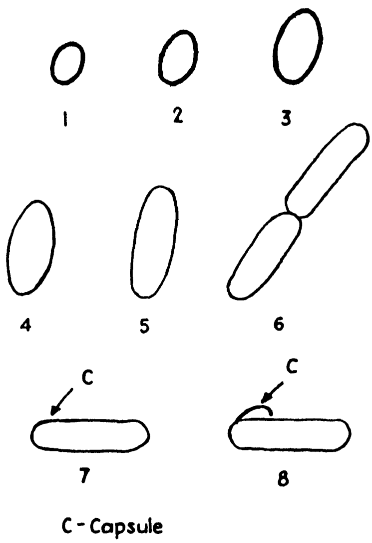


Fig. 30.—Spore germination by absorption. (*After Lamanna.*)

   C. After the second division of the germ cell, even if a thin capsule originally remains, all traces of the spore coat are gone.

Some strains germinating by absorption regularly show a thin capsule remaining about one end of the growing cell. This would appear as a polar germination

(Fig. 30, 7). In other cases equatorial capsules are seen (Fig. 30, 8). Yet in all instances the spore is considered to germinate by absorption inasmuch as the three characteristics of the method are still adhered to.

None of the common spherical bacteria sporulate. Spore production is limited almost entirely to the rod-shaped organisms. Some spore-bearing species can be made to lose their ability to produce spores (asporogenous). This tendency varies with the species. When the ability of a species to produce spores is once lost, it is seldom regained.

Sporulation is not a process to increase bacterial numbers because a bacterial cell very seldom produces more than one spore. Spores are very resistant to adverse or unfavorable environmental conditions and are a means of keeping a species alive, even for many years.

Gram Stain.—This is probably the most important differential stain employed by the bacteriologist. It is usually considered one of the morphological characters because it differentiates bacteria into two groups: the Gram-positive and the Gram-negative organisms.

It was found by Christian Gram (1884) that when histological sections were stained with aniline oil gentian violet by the method of Ehrlich (1882) and then treated with an aqueous solution of iodine, the stain could be easily removed from the tissue sections by alcohol but not from the bacteria embedded therein. He was working on a new method for staining bacteria in tissues but discovered a new differential stain.

In this method of staining, the bacterial film is covered with a solution of one of the methyl violet dyes and allowed to act for a definite period of time. The methyl violet is poured off and a solution of iodine is added. This is allowed to remain for the same period of time. Next the slide is treated with alcohol until no more of the dye is removed from the film. This is then followed by a counterstain such as dilute carbolfuchsin or safranine.

It has been found that some organisms retain the violet stain and others are readily decolorized by the alcohol and take the counterstain. Those that retain the first stain are spoken of as Gram-positive organisms; those that fail to retain the primary stain but take the counterstain are called Gram-negative organisms. Organisms can, therefore, be placed in either of two groups on the basis of the Gram reaction.

The ability of cells to retain the Gram stain is not a property applicable to all living matter in general, but is confined almost entirely to the yeasts and bacteria. The cells of higher plants and animals do not retain the primary stain. Molds stain somewhat irregularly. Granules present in the mycelia tend to retain the stain. The Gram reaction is not a hard and fast one. It may change with the age of the culture, with the pH of the medium, and perhaps in other ways. The change of Gram character with age is especially true of those organisms which are

only weakly Gram-positive and are cultivated in media containing fermentable substances that become acid in reaction as growth proceeds. The reaction is of value only when the various factors are controlled.

Several theories have been advanced to explain the mechanism of the Gram stain but no single theory apparently explains all the facts. The most plausible one appears to be that advanced by Stearn and Stearn (1923, 1924*a*, 1924*b*). Proteins and amino acids are considered as

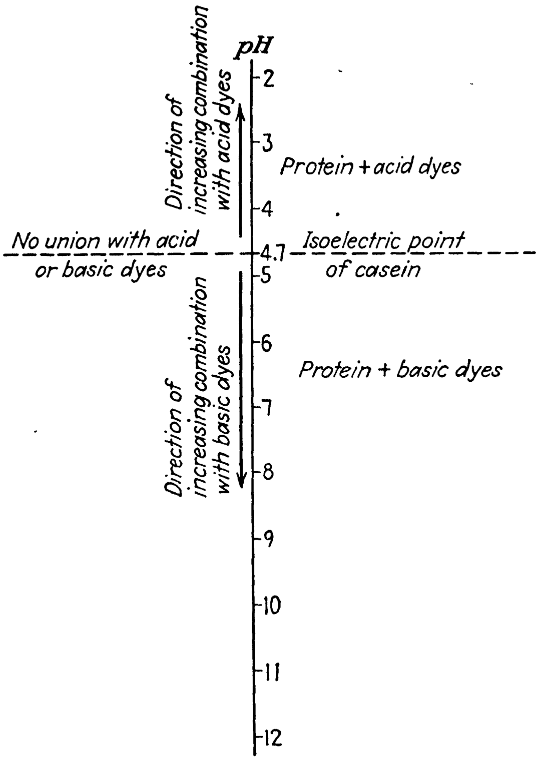


FIG. 31.—Combination of acid and basic dyes with casein, an amphoteric compound.

amphoteric compounds, *i.e.*, have the power to react with both acids and bases by virtue of their carboxyl (COOH) and amino (NH_2) groups. In acid solutions they react with acids; in alkaline solutions they react with bases.

Isoelectric Point.—According to the classical theory the isoelectric point may be defined as the pH where an amphoteric compound shows the least amount of dissociation or, stated differently, it is that point (pH) where the maximum amount of the compound is present in the unionized or molecular state. Opposed to this theory is the newer concept known as the "zwitter ion" hypothesis, which states that the isoelectric point is that pH where the acid and basic groups of the amphoteric compound

are completely ionized (see page 175). On the acid side of the isoelectric point the compound behaves as a base and on the basic side it behaves as an acid.

Basic and acid dyes also combine with proteins. The basic dyes react on the basic side of the isoelectric points and the acid dyes on the acid side of the isoelectric points. The amount of combination in either case is proportional to the degree of alkalinity or acidity of the solutions. At the isoelectric points, proteins combine with neither basic nor acid dyes. Using the protein casein as an example, the action of acid and basic dyes may be schematically represented as shown in Fig. 31.

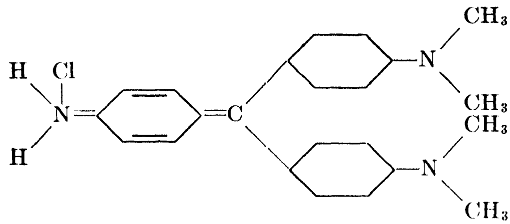
It has been shown that the staining reactions of bacteria are due largely to their protein content. This means that bacteria behave as amphoteric compounds combining with acid dyes in acid solution and basic dyes in basic solution. Combination with either acid or basic dyes does not occur at the isoelectric "range." Since organisms contain more than one protein, the isoelectric point does not have a fairly definite value but rather a series of points extending over two or three pH units. An isoelectric range or zone rather than a point is found.

According to Stearn and Stearn the following conclusions seem justified from their experimental data:

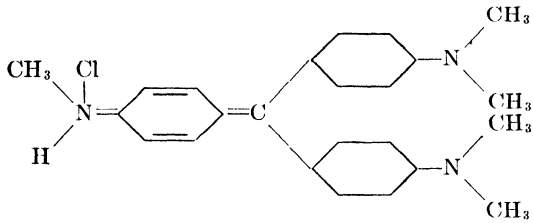
1. Gram-positive organisms can be rendered Gram-negative by increasing acidity.
2. Gram-negative organisms can be rendered Gram-positive by increasing alkalinity.
3. Acid dye-positive organisms can be rendered Gram-negative by increasing alkalinity.
4. Basic dye-positive organisms can be rendered Gram-negative by increasing acidity.
5. At the isoelectric range there is little tendency for any stain to be retained. This range is characteristic of each species.
6. There appears to be good evidence that the proteins of bacteria are not simple proteins but a loose combination of proteins with lipoidal or fatty substances. An example of such a fatty substance is lecithin and a combination of lecithin and protein is known as lecithoprotein.
7. The lipoidal material extracted from Gram-positive organisms differs from that extracted from Gram-negative organisms in that the former contains a much larger proportion of unsaturated acids that have a great affinity for oxidizing agents. All mordants (such as iodine) used in the Gram stain are oxidizing agents. Their effect is in general to render the substance oxidized more acid in character. This increases the affinity of an organism for basic dyes.
8. The change of Gram character with age is especially true of those organisms which are only weakly Gram-positive and are cultivated in media containing fermentable substances which become acid in reaction as growth proceeds.

The *p*-rosaniline dyes give the best results in the Gram stain. The members of this group that have been used more than any of the others include methyl violet, crystal violet, and gentian violet.

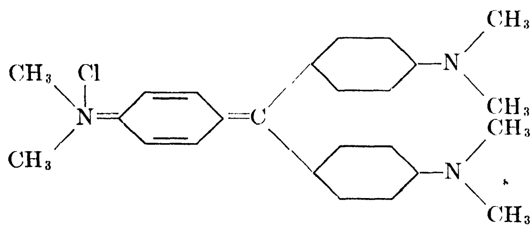
Strictly speaking, methyl violet is the name given to the tetramethyl *p*-rosaniline compound. Commercially the name is usually applied to various mixtures of the tetra-, penta-, and hexamethyl *p*-rosanilines. Their structural formulas are as follows:



Tetramethyl p-rosaniline
(methyl violet)



Pentamethyl *p*-rosaniline



Hexamethyl *p*-rosaniline
(crystal violet)

The shade of color of *p*-rosaniline is deepened by increasing the number of methyl groups in the molecule. Hence, hexamethyl *p*-rosaniline (crystal violet) is deepest in shade and tetramethyl *p*-rosaniline lightest of the three compounds. The names methyl violet 3*R*, 2*R*, *R*, *B*, 2*B*, 3*B*, etc., refer to the number of methyl groups present. The letter *R* denotes the red shades; the letter *B* refers to the blue shades. The most completely methylated *p*-rosaniline is the hexamethyl compound, which is known as crystal violet.

The term gentian violet does not refer to a definite chemical compound but to a poorly defined mixture of violet *p*-rosanilines. The commercial gentian violets are composed largely of the higher methylated compounds having a shade of color at least as deep as that recognized as methyl violet 2*B*.

Crystal violet is also known as methyl violet 10*B*, gentian violet, hexamethyl violet, etc. It produces the deepest shade of any of the *p*-rosanilines and is considered the most satisfactory dye for the Gram technique.

Acid-fast Stain.—The great majority of bacteria are easily stained by the usual bacteriological stains. There are, however, a few notable exceptions. Some bacteria are said to be surrounded by a covering of waxy and fatty substances. These organisms are not penetrated readily by stains but, when once stained, they retain the color even though treated with alcohol containing acid. The organisms are termed

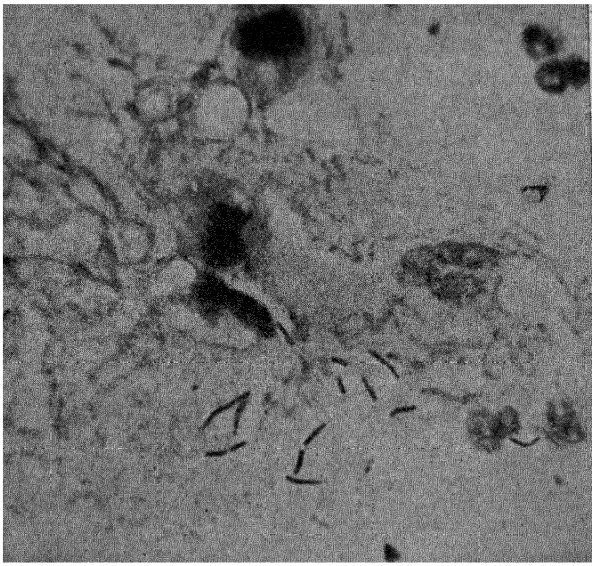


FIG. 32.—Acid-fast stain of *Mycobacterium tuberculosis* in sputum.

acid-fast because they are resistant to decolorization with acid alcohol. The two best-known members of the acid-fast group are the organisms causing tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*M. leprae*).

The acid-fast method for staining the organism of tuberculosis was first announced by Ehrlich in 1882. He stained smears with methyl violet to which was added aniline oil as an intensifier. The organisms may be stained either by long exposure to the dye in the cold or by gentle steaming of the dye on the slide for a shorter period. Ehrlich found that the tubercle bacillus was not decolorized when treated with 30 per cent nitric acid but tissues and other bacteria lost their color. He used vesuvin as a counterstain for the aniline oil methyl violet. By this method the acid-fast organisms appeared purple while everything

else on the slide stained brown. Later Ziehl (1882) improved the keeping qualities of the solution by substituting phenol or carbolic acid for the aniline oil. Neelsen (1883) used carbolfuchsin in place of the aniline oil methyl violet and decolorized the smears with sulfuric acid instead of nitric acid. This modification, with several improvements, is now known as the Ziehl-Neelsen method but is essentially that of Ehrlich.

For staining acid-fast [illegible] distinctly better results will be obtained with new fuchsin than with *p*-rosaniline or rosaniline. The deeper the shade of dye used, the better will be the degree of differentiation.

For an excellent discussion of the cytology of bacteria see the monograph by Lewis (1941).

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CHAPTER V

YEASTS

The yeasts are spherical, oval, or rod-shaped organisms, which grow permanently as single cells and display the simplest possible structure (Fig. 33).

Under certain conditions they may reproduce by elongation of their cells, forming structures having the appearance of a mycelium, but these never show the picture of a true fungus (Fig. 34). In this respect yeasts

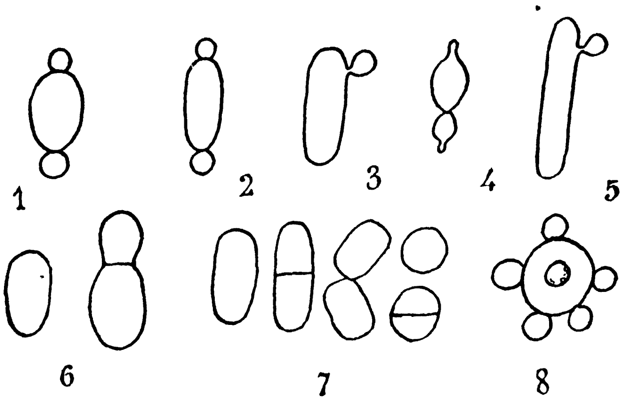


FIG. 33.—Various types of yeast cells. 1, *Saccharomyces cerevisiae;* 2, *S. ellipsoideus;* 3, *S. pastorianus;* 4, *S. apicule;* 5, *Mycoderma;* 6, *Saccharomycodes;* 7, *Schizosaccharomyces;* 8, *Torula.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

differ from molds, which develop mycelia. On the other hand, many molds may temporarily grow as single cells, taking on the appearance of yeasts. Many of the mucors grow as single cells, capable of budding under reduced oxygen tensions. The organisms causing sporotrichosis, coccidioidal granuloma, and blastomycosis appear as single cells in the tissues of the host, whereas mycelia appear on artificial culture media. Members of the *Monilia* grow as single cells on artificial media, but mycelia develop under reduced oxygen tensions (partial anaerobic conditions).

The fact that molds display dimorphism has led some investigators to believe that the yeasts were at one time mold-like but have permanently lost the ability to produce a mycelium. Fuchs (1926) grew the mold *Aspergillus oryzae* under reduced oxygen tension and noted that

yeast-like cells developed from the conidia that were submerged in the medium. A slight fermentation occurred, which resulted in the formation of ethyl alcohol. The cells failed to revert to the mycelial stage but grew permanently as yeasts, even under aerobic conditions.

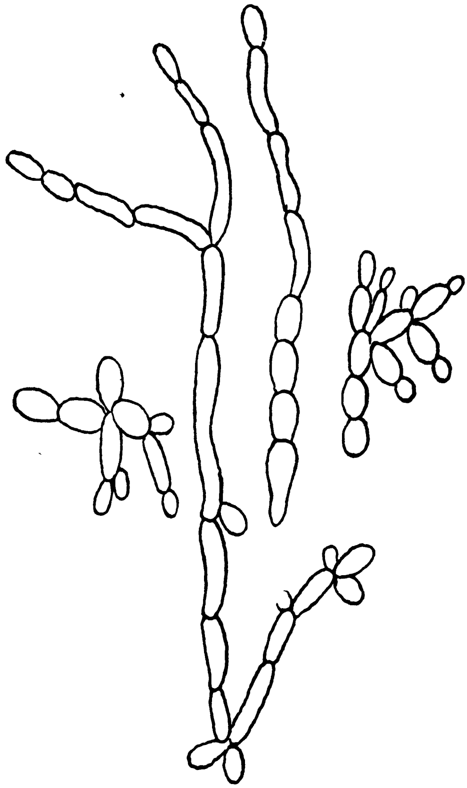


FIG. 34.—Yeast mycelium. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

CYTOLOGY OF YEASTS

Examination of yeasts under the microscope reveals the presence of a cell wall and a relatively large nucleus with which is associated a vacuole containing metachromatic granules.

Cell Wall.—The individual cell is bounded by a delicate membrane, which is relatively thin in young cells and becomes thicker in the more mature cells. The cell wall is composed of a carbohydrate known as yeast cellulose, although different from the cellulose found in the cell walls of higher plants.

Special staining methods reveal the presence of a cytoplasmic membrane, which is surrounded by the cell wall. This membrane cor-

responds in function to that found in bacterial cells (page 50). It is believed to function as a semipermeable membrane in determining which substances may enter and leave the cell.

Granules.—The cell contents of yeasts are more clearly differentiated than those of bacteria. Young yeast cells have a very thin cell wall and relatively homogeneous cytoplasm. As the cells become older, vacuoles and granules appear in the cytoplasm. The granules are known as metachromatic granules or volutin and take an intense stain with methylene blue and other basic dyes, indicating that their composition is very similar to that of the nucleus. Later larger vacuoles are produced, which may be crowded with granules giving a glycogen-like (starch-like) reaction with iodine. Finally the glycogen granules may disappear, leaving the large vacuoles. Also the cytoplasm may show the presence of oil globules. In old cells the cytoplasm and nucleus usually make up only a small proportion of the cellular contents.

Metachromatic granules are composed of either free nucleic acid or a nucleic acid compound and are believed to act as reserve food material for the plant cell. These granules are present in greatest amount in old cells. In young cultures the cells are so active that very little volutin is able to accumulate. As the cells become older and less active, the granules appear. When old cells containing a large number of volutin granules are transferred to a new medium, the granules disappear only to reappear as the cells become old.

Vacuoles.—Vacuoles are structures that are especially characteristic of plant cells and protozoa but occur also in the cells of higher animals. In plant cells, the vacuoles contain a fluid known as the cell sap, which is commonly an aqueous solution of various organic acids and their salts. In protozoa they may contain secretions of the protoplasm or substances about to be excreted, or food in various stages of digestion and assimilation.

Nucleus.—Yeast cells differ from the true bacteria in that they are usually much larger and possess well-defined nucleuses. The nucleus is usually round, sometimes kidney-shaped, owing to compression by a neighboring vacuole, and is situated near the center of the cell. It is difficult to see in hanging-drop preparations but may be seen in stained smears. Some believe that the nucleus possesses the power of ameboid movement through the cytoplasm. This has been observed in connection with the process of budding. After the bud has started to form, the nucleus wanders to that side of the cell and begins to elongate in the form of a dumbbell with one end passing into the bud. Finally the portion in the bud pinches off from the parent nucleus to form two nucleuses, one for the mother cell and one for the bud.

MULTIPLICATION IN YEASTS

Yeasts multiply by budding, by fission, by asexual spore formation, and by sexual multiplication. However, the usual type of multiplication observed is by budding. Sometimes chlamydospores are produced but, since only one spore is produced in a cell, they are not a means of increasing the numbers of yeast cells but a method of perpetuating the species.

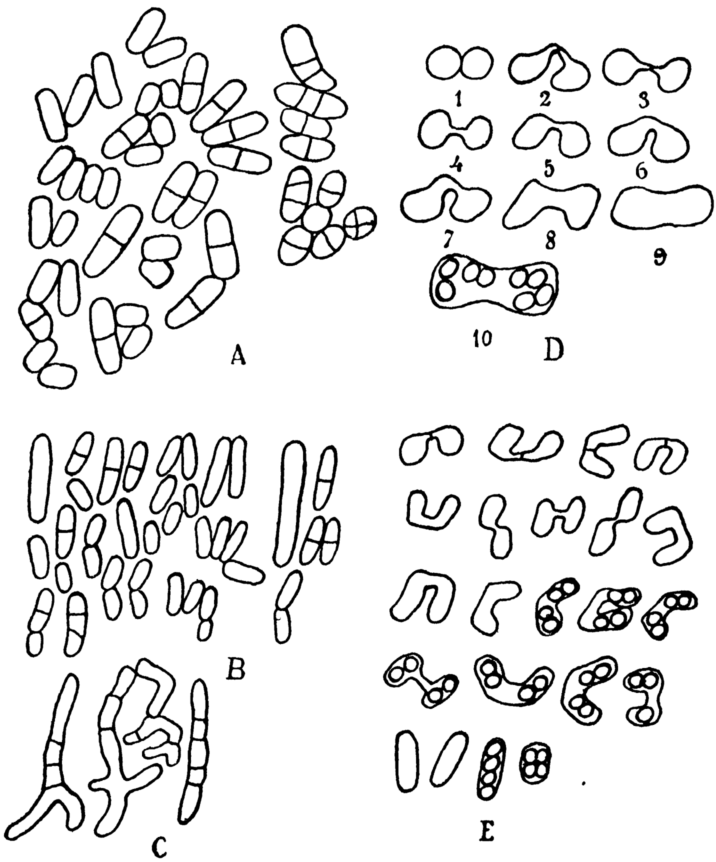


FIG. 35.—*Schizosaccharomyces.* *A, B,* multiplication by transverse fission; *C,* mycelial growth; *D, E,* formation of asci by isogamic copulation. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

Budding.—In almost all the yeasts division of the cell takes place by budding. Budding occurs after the organism acquires a certain definite size. The bud appears as a small prominence separated from the wall of the mother cell by a very narrow collar (Fig. 33). The bud gradually enlarges until it finally separates from the mother cell. As a rule it never reaches the same size as the mother cell before separation.

During periods of rapid division buds may be formed at different points on the surface of the cell. The daughter cells likewise bud at different points before separation from the mother cell occurs. This results in the formation of a small colony or of a chain of yeast cells (Fig. 34).

Transverse Fission.—There are a few species in which multiplication occurs by transverse fission. These yeasts resemble the bacteria in their mode of division.

In *Schizosaccharomyces octosporus* the round or oval-shaped cells elongate to a certain size and then form cross walls in the middle. The

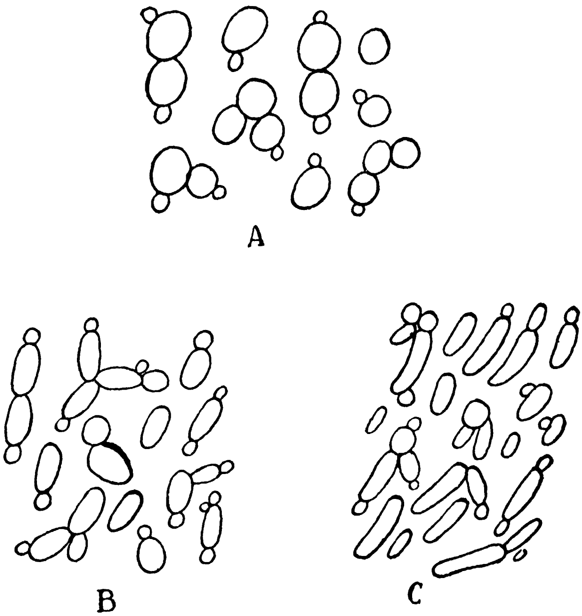


FIG. 36.—*Saccharomyces. A, S. cerevisiae; B, S. ellipsoideus; C, S. pastorianus.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

two cells pull apart and the ends become rounded. When the two new cells reach maturity, they elongate and repeat the cycle. During periods of rapid multiplication cells divide without separating. Transverse partitions also form in the new cells. In this manner a chain of cells is produced resembling a mycelium, which eventually breaks apart (Fig. 35*A*, *B*).

In the genus *Saccharomyces* a form of division intermediate between budding and fission occurs. The cells produce buds generally at their extremities. The cells first elongate and a tube puffs out at one end. This enlarges and is slowly transformed into a bud, which remains attached to the cell by a collar. Finally a wall is formed, which separates the cell from the bud (Fig. 36).

Spore Formation.—Although budding is the usual process of multiplication, such a method does not perpetuate the species. The usual process for perpetuating the species is by spore formation, which is a form of resistance that permits yeast cells to remain viable after budding has stopped. Sporulation may be observed in old cultures where the environmental conditions have become unfavorable for the growth of vegetative cells. Spores are easily produced by inoculating yeasts into a culture medium deficient in nutrients (starvation). When the environment again becomes favorable, the spores germinate into vegetative cells (Figs. 37 and 38).

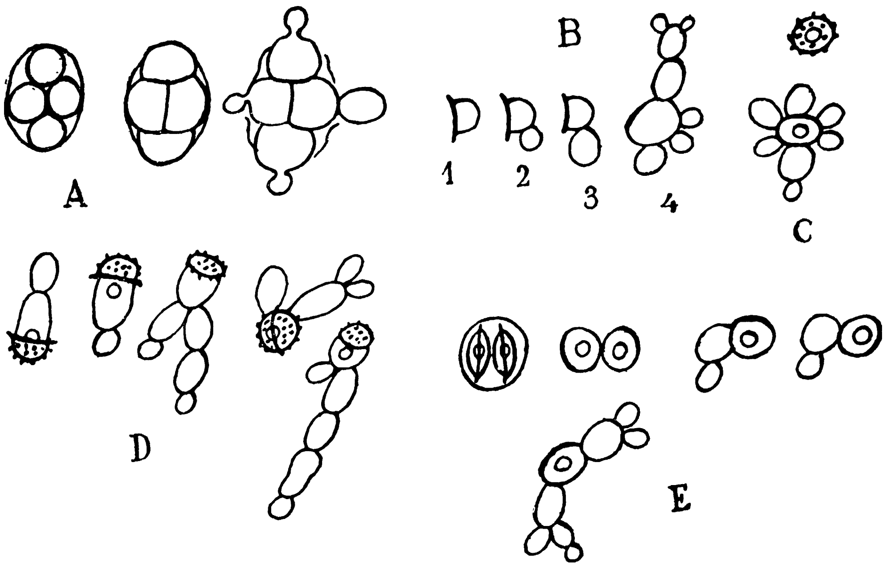


FIG. 37.—Germination of ascospores. *A*, *Saccharomyces cerevisiae; B*, *Willia anomala; C*, *Debaromyces; D*, *Schwanniomyces; E*, *Willia saturnus.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

A definite number of spores is usually produced in the cells of each species. The cells bearing spores are called asci (singular, ascus) and the spores are known as ascospores. In the majority of species the spores are formed by a division of the nucleus twice, giving rise to four spore nucleuses. The nucleuses are surrounded by some cytoplasm around which are formed the spore walls. In other species the number of spores in a cell may be 1, 2, more rarely 8 and 16.

The spores assume various shapes and some species may be easily recognized by this character. Yeast spores are usually round or oval (Fig. 39*A*). Such spores are found in *Saccharomyces cerevisiae,* the common baker's or brewer's yeast, and in other less known species. The spores in *Willia anomala* and in the genus *Hansenia* are hemispherical

and their adjacent surfaces are provided with a projecting border giving them the appearance of a hat (Fig. 39*B*). In the species *Pichia membranaefaciens* the spores are irregularly shaped into oval, elongated,

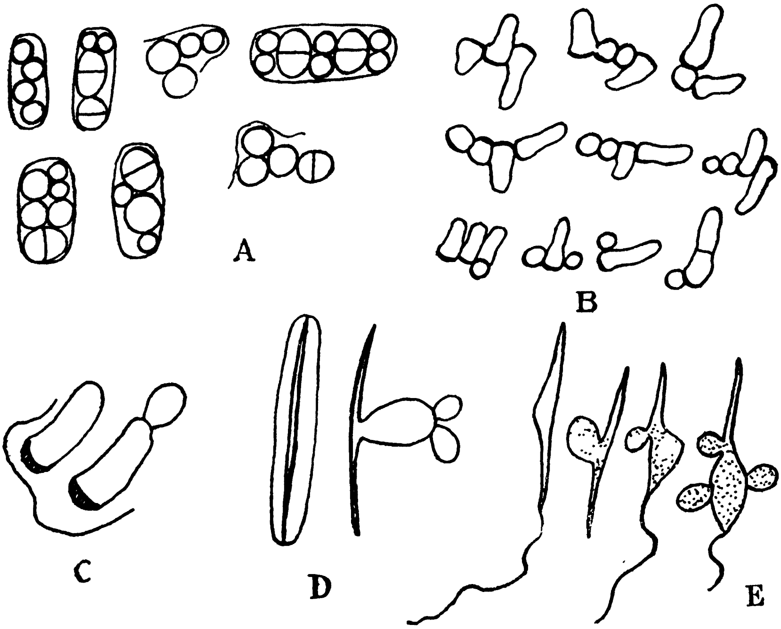


FIG. 38.—Germination of ascospores. *A*, *Schizosaccharomyces octosporus;* *B*, *Schizosaccharomyces pombe;* *C*, *Saccharomycopsis;* *D*, *Monospora;* *E*, *Nematospora.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

FIG. 39.—Various types of ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

triangular, kidney-shaped, or hemispherical forms (Fig. 39*C*). Cells of *W. saturnus* produce spores that are lemon-shaped and are surrounded by a projecting ring (Fig. 39*D*). In the species *Schwanniomyces occidentalis* the spores are surrounded by a projecting ring and the membrane is covered with stiff erect protuberances (Fig. 39*E*). The spores of

Debaromyces are globular and are also covered with protuberances (Fig. 39*F*). Other shapes are illustrated in Fig. 39*G,H,I*.

Sexual Multiplication.—Guilliermond (1920) showed that the asci of *Schizosaccharomyces octosporus* result from the fusion of two cells. When the cells cease to multiply, copulation commences. Two identical cells lying adjacent in a colony are joined by means of a copulation canal (Fig. 40). The two cells are now known as gametes. The wall that

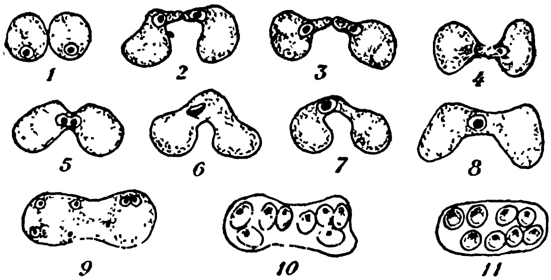


FIG. 40.—*Schizosaccharomyces octosporus.* Isogamic copulation of two cells to form an ascus containing eight ascospores. (*Reprinted from Guilliermond-Tanner, The Yeasts, John Wiley & Sons, Inc.*)

separates the two cells quickly disappears and the nucleus of each passes through the copulation canal. This results in the formation of a single cell or zygospore. The zygospore is formed by isogamic copulation or conjugation. The zygospore increases in size followed by a division of the nucleus into four or eight separate nucleuses. The nucleuses surround themselves with protoplasm. The zygospore now becomes an ascus.

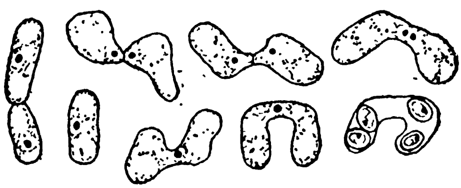


FIG. 41.—*Schizosaccharomyces pombe.* Copulation and incomplete fusion of two cells to form an ascus containing four ascospores. (*Reprinted from Guilliermond-Tanner, The Yeasts, John Wiley & Sons, Inc.*)

In *S. pombe* copulation occurs as in *S. octosporus* except that fusion remains usually incomplete. Copulation takes place between two adjacent cells in the same colony. The gametes are joined by means of a canal through which nuclear and protoplasmic fusion occurs. The nucleus resulting from the fusion quickly divides and the two nucleuses migrate to both enlargements of the zygospore. The nucleuses undergo a second division, resulting in the formation of four spores. The zygospore

becomes an ascus and the spores are known as ascospores (Figs. 35 and 41).

Sometimes it is possible to see a fusion between a cell and an undeveloped bud on the latter. This may be observed in the species *Zygosaccharomyces priorianus*. The ascus that develops is composed of two unequal enlargements, the larger representing the mother cell and the other the bud. Because of lack of space in the bud, the spores

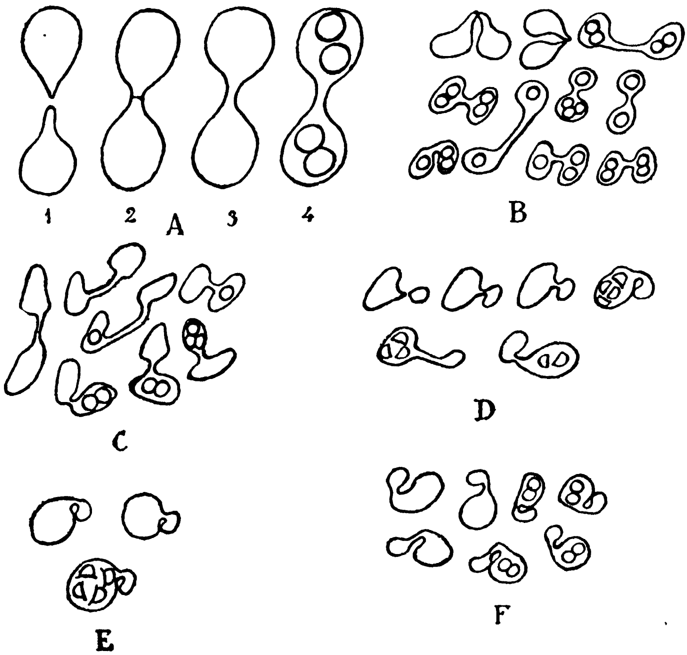


FIG. 42.—*Zygosaccharomyces*. *A*, *B*, ascus produced by isogamic copulation; *D*, *E*, *F*, ascus produced by heterogamic copulation; *C*, ascus produced by copulation intermediate between isogamic heterogamic. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

develop in the mother cell. This is known as heterogamic copulation (Fig. 42*D*, *E*, *F*).

A rarer form of copulation, intermediate between isogamy and heterogamy, has been observed. In these yeasts the two cells, or gametes, are of the same dimensions and do not show any sexual differentiation. After fusion takes place the contents of one cell pass into the other. The former may be regarded as the male cell and the latter as the female cell. The ascospores originate from the female cell and are always two in number (Fig. 42*C*).

In the genus *Nadsonia* copulation occurs by heterogamy between an adult cell and one of its buds. After the two cells fuse, the contents of the male gamete or bud pass into the female gamete or mother cell. A

new cell then forms by budding, and into it pass the contents of the mother cell. This new cell now becomes the ascus and it contains usually only a single ascospore (Fig. 43).

Copulation of Ascospores.—In some species, such as in *Saccharomycodes ludwigii*, *S. johannisbergensis*, and *Willia saturnus*, an isogamic

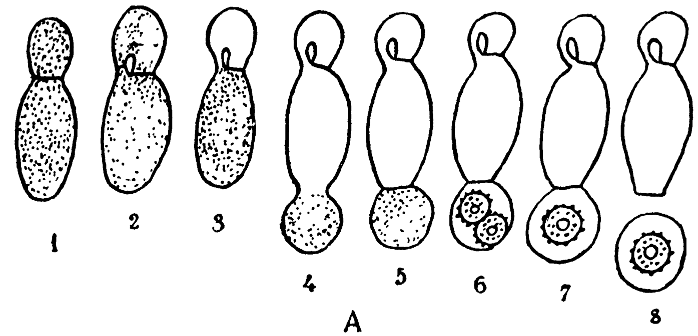


Fig. 43.—*Nadsonia*. Formation of an ascus by heterogamic copulation. (*From Guilliermond's clef dichotomique pour la determination des levures, courtesy of Librairie le François, Paris.*)

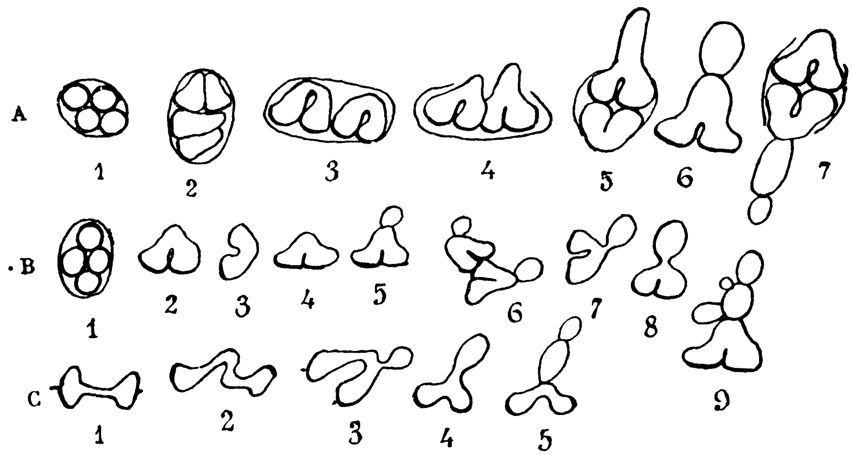


Fig. 44.—Conjugation of ascospores. *A*, *Saccharomycodes ludwigii*; *B*, *Saccharomyces johannisbergensis*; *C*, *Willia saturnus*. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

copulation occurs between the ascospores originating from an ascus. This is shown in Fig. 44. In the species *S. ludwigii* an ascus contains usually four spores. The ascospores copulate, two by two, by means of a copulation canal. This is a true copulation and is accompanied by a fusion of the nuclear material. The fusion remains incomplete and a zygospore is formed from each pair of ascospores united by a copulation canal. The fusion of the two spores results in the formation of a zygo-

spore. It elongates into a germination tube from which develop numerous vegetative cells.

Copulation occurs normally between two spores in the same ascus. It has also been observed between ascospores from different asci and even those more distantly related. This may be observed in old asci in which

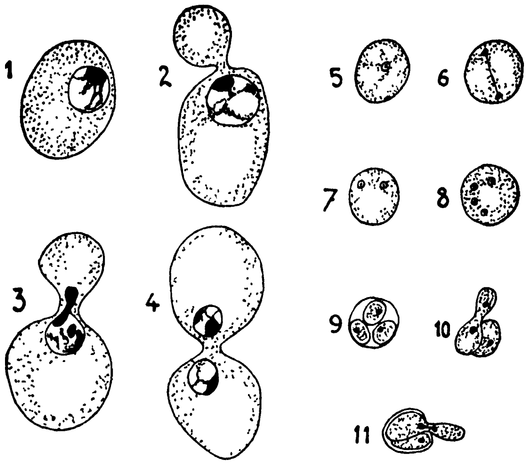


FIG. 45.—*Saccharomyces cerevisiae.* 1–4, formation of a bud with division of the nucleus. 5–11, formation of an ascus from the bud and germination of an ascospore. (*From Gäumann and Dodge, Comparative Morphology of Fungi.*)

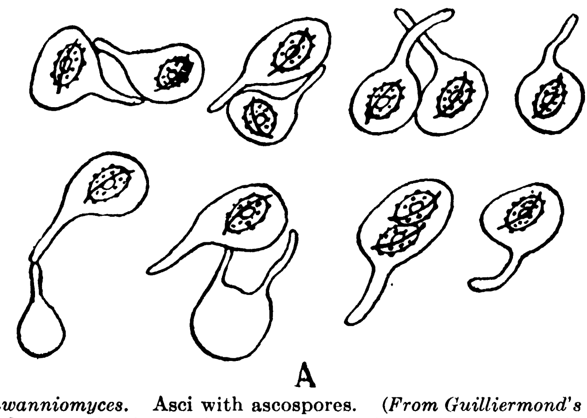


FIG. 46.—*Schwanniomyces.* Asci with ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

many of the spores are dead. Some of the spores can germinate alone; others must fuse in pairs before this occurs. This means that some of those in the latter group are forced to fuse with spores from different asci. The fusion of ascospores is not regarded as a true copulation but as a new process that takes the place of normal sexual fertilization.

Parthenogenesis.—In the great majority of yeasts and especially in those of industrial importance, sexuality has not been observed. These

yeasts represent parthenogenetic forms derived from primitive sex cells. If the development of the ascus is not the result of copulation, it represents a gamete that has developed by parthenogenesis (Fig. 45). Parthenogenesis may be defined as the reproduction of an organism by means of the development of an unfertilized egg or cell.

In the *Schwanniomyces* the cells forming the asci produce projections of different lengths and attempt to fuse together as in true copulation. However, fusion fails to occur and it appears that the cells have retained

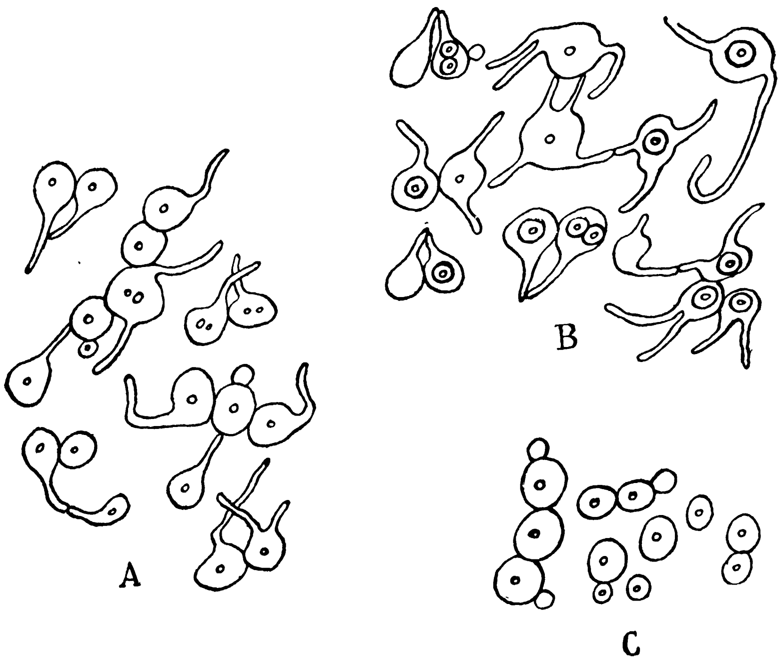


FIG. 47.—*Torulaspora.* (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

only a portion of their sexual characteristics. This same phenomenon has been observed in the *Torulaspora* and in other yeasts (Figs. 46 and 47).

Chlamydospores.—When conditions become unfavorable, yeast cells cease to multiply. Under these conditions some organisms become filled with reserve food products, such as fat and glycogen granules, and enclose themselves in a thick wall. These cells are known as durable cells or chlamydospores. When conditions again become favorable, the chlamydospores germinate into vegetative cells, which in turn multiply by the usual method of budding. Since only one chlamydospore is formed in a cell it is not considered a method of reproduction but rather a method for perpetuating the species.

CLASSIFICATION OF YEASTS

Yeasts have been classified with difficulty and considerable confusion still exists. The classification given here is taken from the works of Stelling-Dekker (1931), Guilliermond (1937), and others.

Family: *Saccharomycetaceae.* Cells single or loosely attached. Asci not differentiated from other cells (see p. 3).

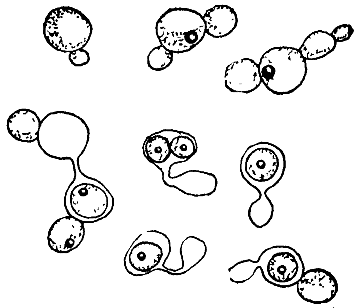


FIG. 48.—*Debaromyces.* (*Reprinted from Guilliermond-Tanner, The Yeasts, John Wiley & Sons, Inc.*)

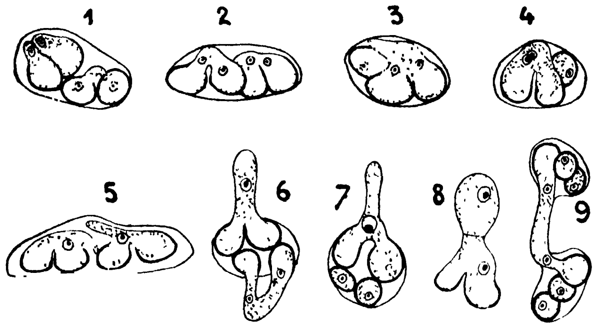


FIG. 49.—*Saccharomycodes.* Asci formed by isogamic copulation of ascospores. (*From Gäumann and Dodge, Comparative Morphology of Fungi.*)

Genus 1. *Zygosaccharomyces.* Cells round, oval, or elongated. Asci formed by isogamic or heterogamic copulation or by a process intermediate between the two. Asci contain from one to four ascospores (Fig. 42).

Genus 2. *Debaromyces.* Cells round or oval. Asci formed by isogamic or heterogamic copulation and contain one or two, sometimes four ascospores (Fig. 48).

Genus 3. *Nadsonia.* Cells round, oval, elliptical, or lemon-shaped. Asci derived by budding from a cell formed by heterogamic copulation. One or two, sometimes up to four round, verrucose ascospores produced in each ascus (Fig. 43).

Genus 4. *Schwanniomyces.* Cells round or oval, occasionally show rudimentary mycelia. Asci formed by parthenogenesis. One or two ascospores produced in each ascus. Spores provided with a projecting collar and with a verrucose wall (Fig. 46).

Genus 5. *Torulaspora.* Cells round with a large, fat globule in the center. Asci show a trace of copulation by development of projections but union does not

occur. Asci produced by parthenogenesis. One or two round and smooth ascospores observed in each ascus (Fig. 47).

Genus 6. *Saccharomycodes.* Asci contain four round, smooth spores. Ascospores conjugate by isogamic copulation resulting in the formation of a zygospore. The zygospore germinates by budding (Fig. 49).

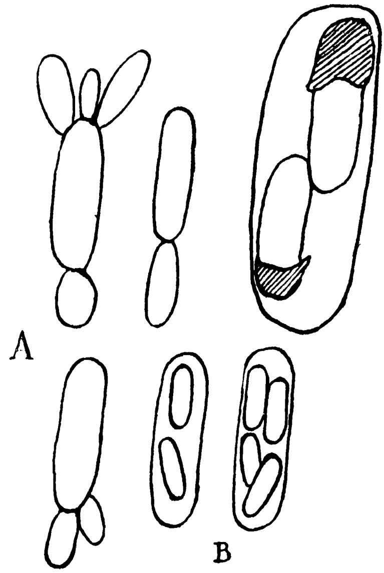


FIG. 50.—*Saccharomycopsis.* Ascospores provided with a double membrane. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

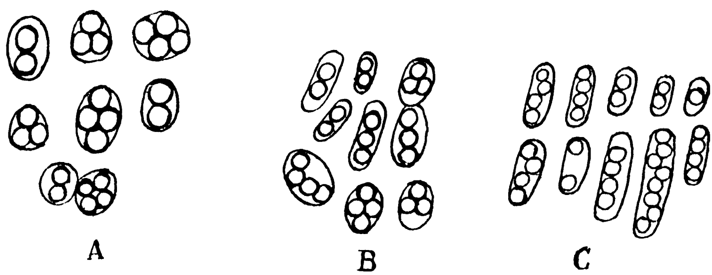


FIG. 51.—*Saccharomyces* Asci and ascospores. *A*, *S. cerevisiae*; *B*, *S. ellipsoideus*; *C*, *S. pastorianus.* (*From Guilliermond's clef dichotomique pour la détermination des levures courtesy of Librairie le François, Paris.*)

Genus 7. *Saccharomycopsis.* Cells large, oval, or rectangular and are often united into groups at their ends. Budding occurs at both ends of the cells. Asci contain one to four ascospores. Spores provided with a double membrane (Fig. 50).

Genus 8. *Saccharomyces.* Cells round, oval, or elongated. Asci formed without conjugation and contain one to four round, smooth ascospores. Spores germinate by budding (Figs. 45, 51).

Genus 9. *Hansenia.* Cells lemon-shaped. Multiplication occurs by budding and by the formation of asci without conjugation. Asci contain from one to four spores showing a projection ring or collar (Fig. 52).

Genus 10. *Pichia.* Cells oval or elongated. Asci formed by isogamic or heterogamic conjugation or by parthenogenesis. Asci contain one to four round, hemispherical, or triangular ascospores (Fig. 53).

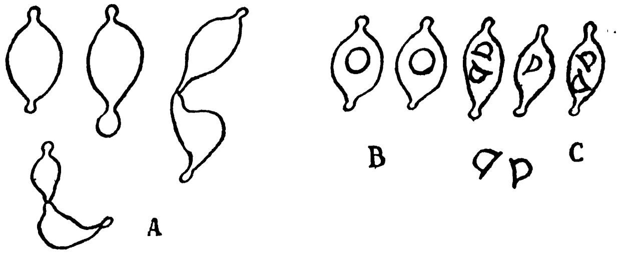


FIG. 52.—*Hansenia.* *A*, cells; *B*, *C*, asci and ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

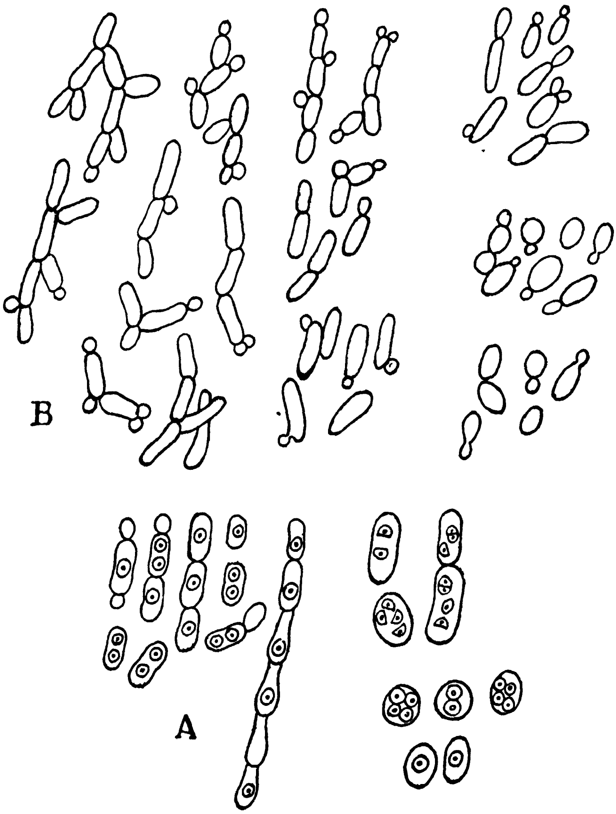


FIG. 53.—*Pichia.* *A*, asci and ascospores. *B*, budding cells. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

Genus 11. *Willia.* Cells oval or elongated, occasionally round. Asci formed without conjugation and contain from one to four ascospores. Spores lemon-shaped or hat-shaped. Spores germinate by budding (Fig. 54).

Genus 12. *Monospora.* Cells oval-shaped and reproduce by budding. Asci contain only one needle-shaped ascospore. Spores germinate by budding (Fig. 55).

Genus 13. *Nematospora.* Cells of variable form and reproduce by budding. Asci contain 8 or 16 needle-shaped motile spores provided at one of their poles with a flagellum. Spores germinate by budding (Fig. 56).

Genus 14. *Coccidiascus.* Cells oval-shaped and reproduce by budding. Asci formed by isogamic conjugation and contain four spindle-shaped ascospores (Fig. 57).

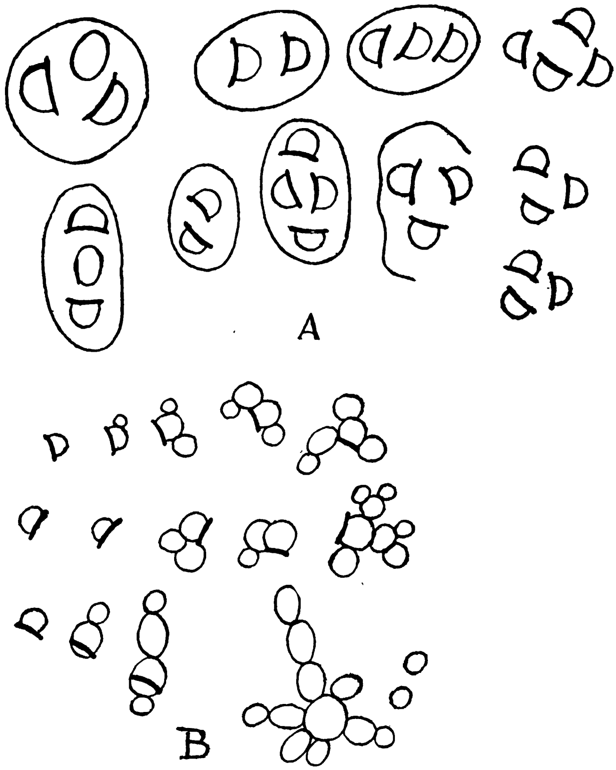


FIG. 54.—*Willia.* *A*, asci and ascospores; *B*, germination of ascospores by budding. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

Torulae.—The term Torulae (singular, Torula) is generally applied to the species of yeasts that are not capable of producing spores. They are sometimes named false or wild yeasts. Will (1916) placed all of the nonsporing yeasts in the family Torulaceae and Harrison (1928) divided it into four genera as follows:

Family: *Torulaceae.*

Genus 1. *Rhodotorula.* Cells produce a red pigment.

Genus 2. *Chromotorula.* Cells produce pigments other than red.

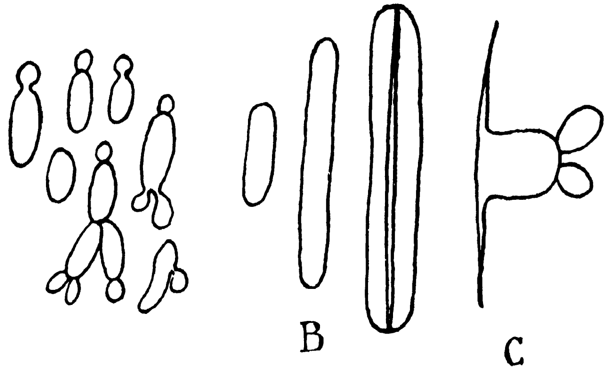


FIG. 55.—*Monospora.* *A*, budding cells, *B*, ascus with one ascospore, *C*, germination of ascospore by budding. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

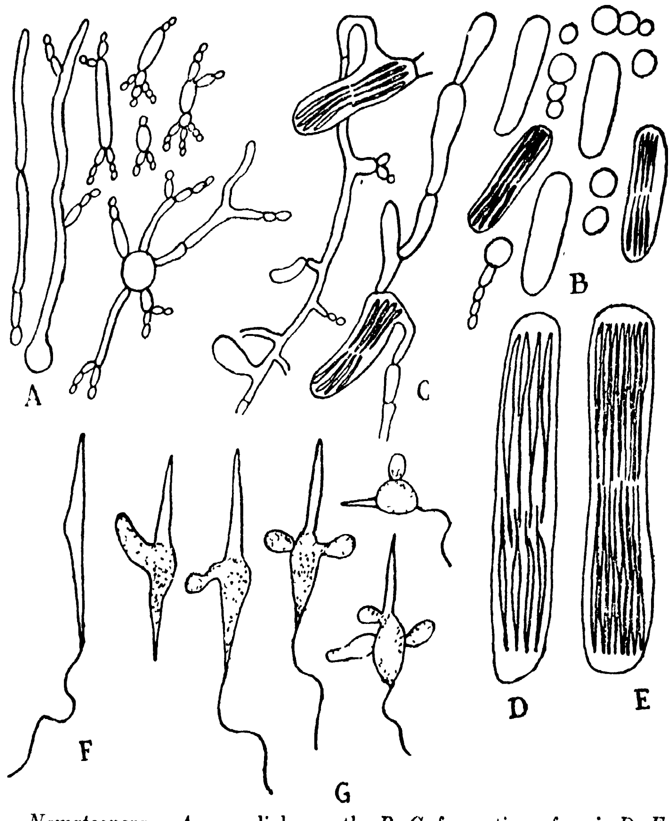
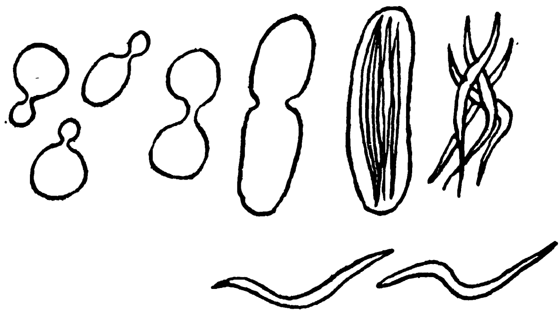


FIG. 56.—*Nematospora.* *A*, mycelial growth; *B*, *C*, formation of asci; *D*, *E*, asci filled with 8 or 16 ascospores. *F*, *G*, germination of ascospores. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Librairie le François, Paris.*)

Genus 3. *Mycotorula.* Cells do not produce a pigment. Hyphae formed.
Genus 4. *Torula.* Cells do not produce a pigment. Hyphae not formed.

Saccharomyces.—Since this group contains all the species of industrial importance, it is by far the most important genus of the yeasts. The members reproduce by budding and by asexual spore formation. Sexual multiplication has not been observed in any of the species. The best known member is *S. cerevisiae,* the common species used in the baking and brewing industries. It is the same species that is employed medicinally and for the fermentation of carbohydrate solutions to alcohol and carbon dioxide.

Pityrosporum.—The yeast-like organism known as *Pi. ovale* is usually referred to as the bottle bacillus because of its resemblance to a bottle.



A

FIG. 57.—*Coccidiascus.* Ascus produced by isogamic copulation. Four ascospores in each ascus. (*From Guilliermond's clef dichotomique pour la détermination des levures, courtesy of Libraire le François, Paris.*)

It has been isolated from the scalp and for that reason is accused by some of being the etiological agent of dandruff. However, there is no evidence that it has anything to do with the formation of dandruff or any pathological condition of the scalp.

HYBRIDIZATION

A hybrid may be defined as the development of a new organism from two cells of different species or different genetic make-up.

Winge and Laustsen (1938, 1939) showed that it is possible to breed new varieties of yeasts by hybridization. They placed two spores from different species in a drop of culture solution so as to enable them to copulate to form a zygospore from which the hybrid yeast germinated The copulation was observed under the microscope. These workers succeeded in producing 14 new yeast types from 8 different species and strains of *Saccharomyces* and one of *Zygosaccharomyces.*

They observed that the ability of a yeast to produce a definite enzyme was always dominant in the hybrid. This means that if one

of the species or strains hybridized elaborates a sucrase and the other does not, the hybrid will always possess the ability to elaborate a sucrase.

Improved yeast types may be produced by hybridization and selection. In spite of the limited number of experiments performed by the above-named workers, one of the hybrids has already been employed commercially with excellent results. Hybridization of yeasts appears to have far-reaching possibilities.

LABORATORY TECHNIQUE

The methods used for the isolation and study of yeasts in pure culture are, in general, the same as those employed for bacteria. Most species of yeasts grow best at a temperature of about 25°C.

Induced Sporulation.—The conditions necessary to induce sporulation include the following:

1. The yeast cells must be young and vigorous.
2. Plenty of moisture and oxygen must be present.
3. The medium must be deficient in nutrients.
4. The organisms must be incubated at a certain temperature, usually 25°C.
5. The medium must possess a suitable hydrogen-ion concentration.
6. Inhibitory substances must be absent from the medium.

One of the earliest methods recommended the use of plaster of Paris blocks. Plaster of Paris is molded into blocks and the upper surface scraped smooth. The block is placed in a glass container, such as a Petri dish, and water added until the lower portion is submerged. The glass container and contents are sterilized in the autoclave. The upper surface of the block is heavily inoculated from a young agar culture and the container incubated at 25°C. for 48 hr. or longer. This method usually produces satisfactory sporulation. Slices of potato or carrot have been substituted for the plaster of Paris block with good results.

Gorodkowa recommended the use of the following medium to induce sporulation:

Glucose	0 25 gm.
Beef extract	1 00 gm.
Agar	1 00 gm.
Sodium chloride	0 50 gm.
Distilled water	100 00 cc.

The medium is tubed, sterilized, and slanted until firm. The surface is then inoculated heavily from a young, vigorous culture of yeast.

An agar medium containing a weak infusion of carrots and a small amount of calcium sulfate has also been employed with good results. Other types of culture media may be employed, provided the nutrients are present in deficient amounts.

Isolation of Pure Culture.—Many methods have been recommended for the isolation of yeasts in pure culture. Most of the methods are concerned with the isolation of a single yeast cell and its propagation in a suitable culture medium. Such methods require considerable skill and patience. Yeasts may also be isolated in pure cultures by the same methods employed for the separation of bacterial species (page 116).

For an excellent discussion on yeasts see the monograph by Henrici (1941).

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CHAPTER VI

MOLDS

Molds are saprophytic or parasitic fungi, which reproduce by means of sexual or asexual spores. They do not contain chlorophyll.

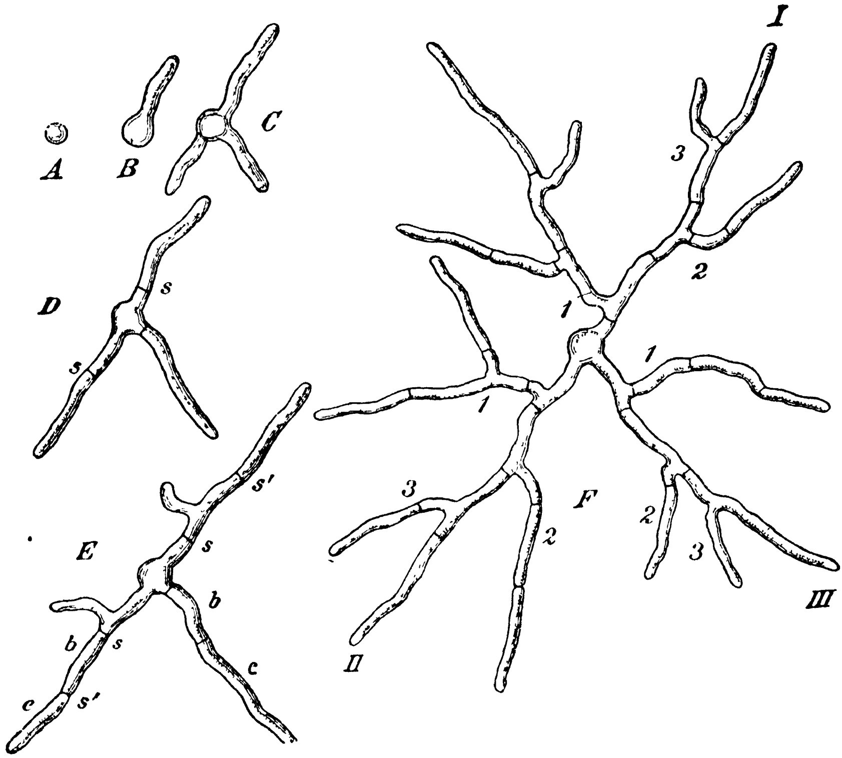


FIG. 58.—*Penicillium glaucum.* Mycelial development. *A*, ripe spore; *B, C, D, E, F,* mycelial growth from single spores. (*After Zopf; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

In most species the molds possess a thallus which is composed of a mass of threads known as hyphae (singular, hypha). The whole mass of threads taken collectively is spoken of as a mycelium (Fig. 58). The filaments or hyphae are usually colorless. Hyphae that are concerned in the production of spores are termed the fertile hyphae; those serving to secure nutrients are called the vegetative hyphae.

Structure of Hyphae.—Hyphae may be single-celled (nonseptate) or divided by transverse walls known as septa (singular, septum). Longitudinal or oblique septa are very rare. Hyphae are more or less branched, continuous tubes. In most molds increase in size of hyphae occurs by apical growth. Cells lying back from the tips of hyphae may start to grow and develop into branches. In a few species all cells may continue to grow and divide. The nonseptate hyphae form one large cell containing many nucleuses. The septate hyphae may contain one, two, or many nucleuses in each cell. Septa are rare in the phycomycetes except in the fruiting bodies though they may be occasionally present in old cultures.

The nucleuses are usually easily differentiated from the remainder of the cytoplasm. Young cells show a relatively dense cytoplasm, whereas older cells become vacuolated. The protoplasm (cytoplasm + nucleus) of mature cells usually makes up only a small portion of the total cell volume. Various reserve food granules, such as fat globules, glycogen and metachromatic granules, or volutin may also appear.

Cell Wall.—The cell wall shows some variation in composition. In many Archimycetes and Oomycetes and in the yeasts the cell wall is composed largely of cellulose, but in the Zygomycetes and higher fungi, the cellulose is replaced by a polymerized acetylated glucosamine known as chitin, and by other substances of an obscure nature. Chitin is also present in the shells of crabs and lobsters and in the shards of beetles.

MULTIPLICATION IN MOLDS

Most of the common molds may be cultivated by transferring any part of a plant to fresh culture medium but the normal process of development begins with the germination of a spore. Spores are produced in great numbers. They are very resistant bodies being capable of withstanding adverse conditions over long periods of time. Spores are of different shapes and sizes and may be composed of one cell or more than one cell.

Two types of spores are produced; sexual and asexual. An asexual spore is one that is not the result of the fusion of two gametes or sex cells. A few of the molds produce several kinds of spores, corresponding to different stages in their development. Practically all the molds commonly encountered produce asexual spores. A few of these produce both sexual and asexual spores.

Sexual Multiplication.—The nonseptate molds produce sexual spores known as zygospores. In zygospore formation two filaments lying near each other send out branches that finally touch. The apical cells of the two branches fuse together. Cell walls are then formed, which

separate the fused mass of protoplasm from the remainder of the hyphae. The resulting cell greatly enlarges in size and develops a very thick and irregular cell wall. The zygospore germinates into a new fungus when conditions are favorable. This is shown in Fig. 59.

The septate molds that produce sexual spores develop ascospores. Ascospores are formed by the development of two cells from either the same or adjoining hyphae. The cells coil together and fuse at their tips resulting in the formation of one cell. The cell grows, branches more or less, and finally becomes surrounded by a mass of threads or hyphae. Sacs develop from the branches of the fertilized cell. These sacs are known as asci (singular, ascus). Usually eight spores develop in each ascus. The spores are known as ascospores.

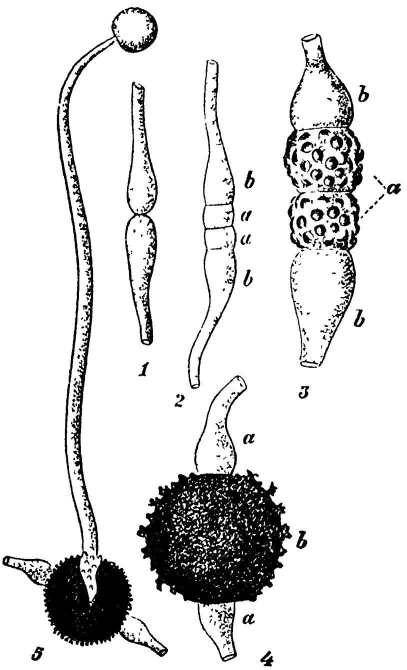


FIG. 59.—*Mucor mucedo.* Formation of zygospore. 1, two hyphae in terminal contact; 2, articulation into gamete *a* and suspensor *b*; 3, fusion of gametes *a*; 4, ripe zygospore *b* supported by the suspensors *aa*; 5, germination of zygospore. (*After Brefeld; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

Asexual Multiplication.—Asexual multiplication in molds takes place in both nonseptate and septate molds. The spores produced by nonseptate molds may be either free or enclosed in spore cases known as sporangia (singular, sporangium). The septate molds never produce sporangia.

A sporangium is produced at the upper end of a fertile hypha. The hypha becomes enlarged at the end, increases in length, and finally consists of a long filament with a swollen tip. The enlarged portion is filled with cytoplasm in which are present many nucleuses. The nucleuses surround themselves with some cytoplasm and develop cell walls to produce spores. These spore cases are known as sporangia. The portion of the hypha reaching into the interior of the sporangium is called a columella. The filament bearing the sporangium is known as a sporangiophore. When the sporangium ripens the wall ruptures, scattering the spores. Each spore is capable of repeating the cycle (Fig. 60).

Molds that do not produce sporangia develop definite fertile threads known as conidiophores. The conidiophores produce asexual spores known as conidia (singular, conidium). Conidia are borne on the tips of

branches in the form of chains. In some species the tip of the conidiophore constricts and pinches off spores. The spore nearest the mother cell is the youngest (Fig. 61). In other species, spores are formed as the result of a budding from the terminal cell of the conidiophore. The

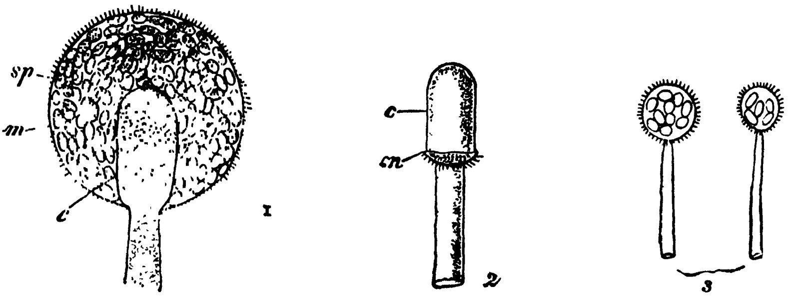


Fig. 60.—*Mucor mucedo.* 1, sporangium; 2, remains of an emptied sporangium; 3, stunted dwarf sporangia, with only a few spores and devoid of columella. (*After Brefeld; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

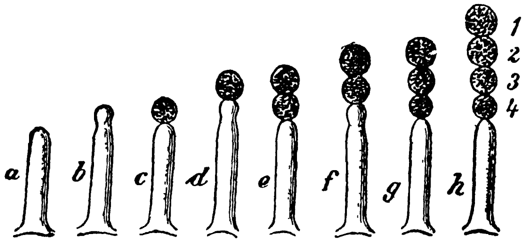


Fig. 61.—Formation of conidia. The topmost (1) is the oldest. (*After Zopf; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

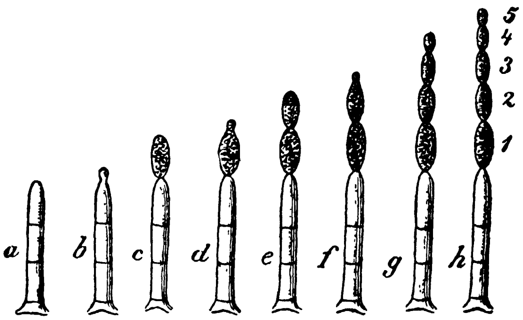


Fig. 62.—Formation of conidia. The topmost (5) is the youngest. (*After Zopf; from Lafar's Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

spore then grows to full size, after which another spore pushes out from the tip of the new spore. This continues until a chain of spores is formed. In these molds the terminal spore of the chain is the youngest (Fig. 62).

Sometimes cells in a filament surround themselves with very thick cell walls to form spores. These are asexual spores and are known as

chlamydospores. They occur either singly or in chains in ordinary vegetative hyphae or in special branches. Chlamydospores are formed by a contraction of the contents of portions of hyphae resulting in the loss of water and the development of thick walls.

In some species the vegetative mycelium breaks up into short segments or spores. These segments break apart and are capable of giving rise to new plants. The segments or spores are called oidia (singular, oidium).

RESISTANCE OF MOLD SPORES

The growth of molds has been responsible for enormous losses in the home and in the industries. These losses are undoubtedly due to the fact that molds produce large masses of spores. Mold spores are very resistant to unfavorable conditions such as heat, cold, desiccation, ultraviolet light, high osmotic pressures, and deficient food supply. Generally speaking, mold spores are more resistant to heat than mycelium and less resistant than bacterial spores.

Spores are easily disseminated by wind and air currents. They are commonly present in the air of laboratories and are the frequent cause of contaminations of cultures and culture media. Therefore, laboratory windows should be kept closed to minimize aerial contaminations.

Molds occur particularly in damp places. The spores will not germinate in a dry environment. Many industrial products such as paper, leather, textiles, and foods readily absorb moisture from a moist atmosphere and are susceptible to attack by molds. In order to decrease mold contaminations, laboratories and rooms should be kept as free as possible from any excessive amount of moisture.

CLASSIFICATION OF MOLDS

The general principles followed for the classification of molds are similar to those employed for the classification of bacteria (page 359). A condensed classification of the commonly encountered genera and species of molds is as follows:

Class I. *Phycomycetes.* Vegetative mycelium, usually nonseptate. Sporangia with motile or nonmotile sporangiospores or conidia produced. Resting sporangia, oospores, or zygospores are formed, sometimes after fertilization, sometimes without any preliminary fusion of gametes or gametangia. Some are parasitic; others are saprophytic.

Order: *Mucorales.* Accessory multiplication by sporangiospores.

Sporangia globose to ovoid, usually contain numerous spores, sometimes one or a few. Zygospores formed from the whole of the two gametangia.

Columella present. Zygospore naked or invested by outgrowths from its own wall or from those of the suspensors.

Principal sporangia contain numerous spores.

Family 16. *Mucoraceae.* Sporangiola, if any, developed on lateral branches of principal sporangiophores. Sporangia are of one kind. Sporangiophore

simple or branched but not repeatedly dichotomous. Suspensors without appendages at maturity.

Genus 1. *Mucor.* Aerial mycelium absent. Sporangia single and terminal.

Sporangiophores rarely or never branched:

Species 1. *hiemalis.* Columella spherical.

Species 2. *piriformis.* Columella pear-shaped.

Species 3. *mucedo.* Columella elongate to ellipsoidal.

Sporangiophores usually branched:

Species 4. *plumbeus.* Columella usually spiny near tip.

Species 5. *rouxii.* Rapidly converts starch to sugar. Used in manufacture of alcohol.

Sporangiophores with main stem and secondary lateral branches, racemose:

Species 6. *racemosus.* Columella ovoid.

Species 7. *erectus.* Columella spherical, sporangium gray yellow.

Species 8. *fragilis.* Columella spherical, sporangium black.

Branches of sporangiophore nearly equal, cymose:

Species 9. *ambiguus.* Sporangia borne irregularly.

Species 10. *circinelloides.* Sporangia in two rows, alternating. Spores spherical to ellipsoidal.

Species 11. *alternans.* Sporangia in two rows, alternating. Spores longer, ellipsoidal.

Genus 2. *Rhizopus.* Aerial mycelium stoloniferous.

Species 12. *nigricans.* The common black bread mold.

Species 13. *oryzae.* Employed in the hydrolysis of starch to sugar.

Species 14. *japonicus.* Also employed in the hydrolysis of starch to sugar.

Class II. *Ascomycetes.* With the exception of the yeasts, all possess a well-developed mycelium of branched and septate hyphae. Cells of mycelium may be uninucleate or may contain several nucleuses. Multiplication takes place by conidia and by chlamydospores but the characteristic method is by means of ascospores. An ascus contains usually 8 spores, more rarely a smaller or larger number.

Class III. *Basidiomycetes.* All possess well-developed mycelium. Basidiospores produced which are borne externally on the mother cell or basidium. The young basidium contains two nuclei that fuse, then divides to provide the nuclei of the spores. The spore is formed on a sterigma through which the nucleus passes from the basidium to enter the developing spore. The basidiospores are unicellular, round or oval, asymmetrically attached to their sterigmata, usually with a smooth, rather thin wall. Echinulate spores occur in a few species.

Class IV. *Fungi Imperfecti.* Characteristic method of multiplication is by means of conidia. In some species oidia and chlamydospores may be present. Sporangiospores, ascospores, and basidiospores are not produced.

Order: *Hyphomycetales.* Conidiophores free, arising irregularly from the mycelium. Conidiophores detached, not compacted.

Family 1. *Mucedinaceae.* Hyphae colorless or in pale or bright colors.

Genus 1. *Trichothecium.* Spores two-celled, in small clusters on ends of erect conidiophores.

Species 1. *roseum.* A commonly occurring pink mold found on decaying fruit. At times causes contamination of laboratory media.

Genus 2. *Oospora (Oidium).* Reproduction by fragmentation of mycelium. Conidia exogenous, globose, or suboblong.

Species 2. *lactis.* Commonly found in milk and cheese. Imparts flavor and aroma to many types of cheeses.

Genus 3. *Monilia*. Spores distinct from mycelium. Reproduction by ovate spores, increasing by budding and forming branched chains, also by fragmentation of mycelium in old cultures.

Species 3. *sitophila*. Commonly found in air. Grows rapidly and is the cause of many laboratory contaminations.

Genus 4. *Aspergillus*. Conidiophores arising from specialized foot cells, usually nonseptate, terminating in a swelling which bears the sterigmata. Spores borne in chains formed by abscission from sterigmata.

Species 4. *candidus*. Spores white or nearly so. Sterigmata unbranched.

Species 5. *albus*. Spores white or nearly so. Sterigmata branched.

Species 6. *glaucus*. Spores green. Sterigmata unbranched. Perithecium naked, not embedded.

Species 7. *clavatus*. Spores colored. Tip of conidiophores only slightly swollen, club-shaped, sterigmata along sides for a considerable distance. Perithecium embedded. Sterigmata unbranched.

Species 8. *fumigatus*. Tip of conidiophore hemispherical, sterigmata unbranched and produced only from terminal portion. Perithecium embedded.

Species 9. *giganteus*. Does not produce perithecia. Tip of conidiophore very large. Sterigmata unbranched.

Species 10. *flavus*. Does not produce perithecia. Tip of conidiophore small. Conidiophore rough and warty. Spores yellow. Sterigmata unbranched.

Species 11. *oryzae*. Conidiophore smooth. Does not produce perithecia. Sterigmata unbranched.

Species 12. *versicolor*. Sterigmata branched. Mycelium rusty brown.

Species 13. *pseudoclavatus*. Tip of conidiophore club-shaped. Sterigmata both lateral and terminal, branched.

Species 14. *nidulans*. Tip of conidiophore hemispherical. Sterigmata terminal, branched.

Species 15. *calyptratus*. Spores black or dark brown. Sterigmata unbranched.

Species 16. *niger*. Spores black. Sterigmata branched.

Species 17. *wenti*. Spores coffee brown. Sterigmata unbranched.

Species 18. *ochraceus*. Spores yellow brown. Sterigmata branched.

Genus 5. *Penicillium*. Conidiophores little or not inflated, unequally verticillate at tip. Conidia globoid, not in mucus.

Species 19. *camemberti*. Found on Camembert or Brie cheese. Floccose colonies, white or grayish green in color.

Species 20. *brevicaule* var. *glabrum*. Found on Camembert or Brie cheese. Powdery colonies, yellowish white in color.

Species 21. *brevicaule*. Found on Camembert or Brie cheese. Yellow brown areas formed. Spores rough.

Species 22. *roquefortii*. Found on Roquefort cheese. Forms green streaks inside of cheese.

Species 23. *italicum*. Found on citrus fruits. Mold colonies are blue green.

Species 24. *digitatum*. Found on citrus fruits. Mold colonies are olive green.

Species 25. *expansum*. Found on apples and pears. Mold colonies are blue green.

Family 2. *Dematiaceae.* Mycelium, spores, or both dark brown to black.

Genus 1. *Cladosporium.* Spores increase by budding, forming branched chains. Spores one-celled but become two-celled in old cultures.

Species 1. *herbarum.* Found on decaying paper, straw, and similar materials.

Genus 2. *Alternaria.* Spores many-celled, club-shaped, and in chains.

Species 2. *tenuis.* Found on moldy grains and in soil. Frequently found in laboratory air.

MORPHOLOGY OF THE COMMON MOLDS

Several hundred genera and thousands of species of molds have been described. Only a few genera are of common occurrence and these may

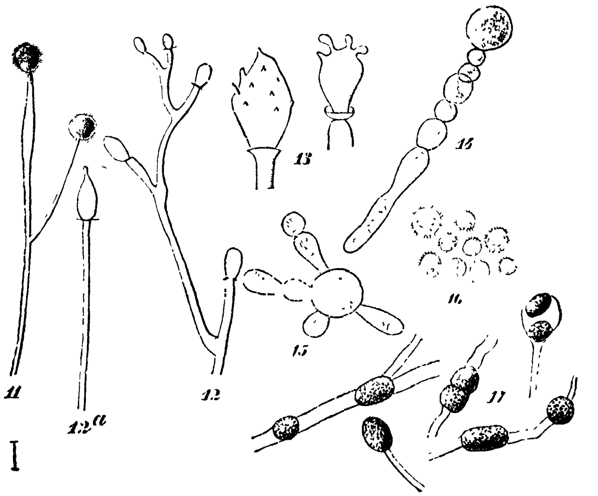


FIG. 63.—*Mucor plumbeus.* 11, sporangiophores and sporangia; 12–12a, sporangiophores and columella; 13, columella; 14, spherical cells; 15, budding spherical cells; 16, spores; 17, chlamydospores. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

be easily recognized. The commonly occurring genera include: *Mucor, Rhizopus, Trichothecium, Oidium* and *Oospora, Monilia, Aspergillus, Penicillium, Cladosporium,* and *Alternaria.* Their characteristics are as follows:

Mucor.—This is the largest genus of the order Mucorales. The species belonging to this genus are found on decaying vegetables and on bread. The vegetative mycelium penetrates the food material and sends out long, slender threads known as aerial hyphae. The mycelium is white in color. A septum forms near the apex of each hypha. The tip of the hypha then swells into a globular sporangium within which develop numerous oval spores. The sporangia are almost black in color. The wall of the ripe sporangium easily breaks, [illegible] the enclosed spores. Each spore is capable of repeating the cycle (Figs. 63, 64, 65,

66, 67, and 68). Under certain conditions conjugation of two cells from different hyphae precedes spore formation, resulting in the development of a zygospore. This is sexual multiplication. A germ tube

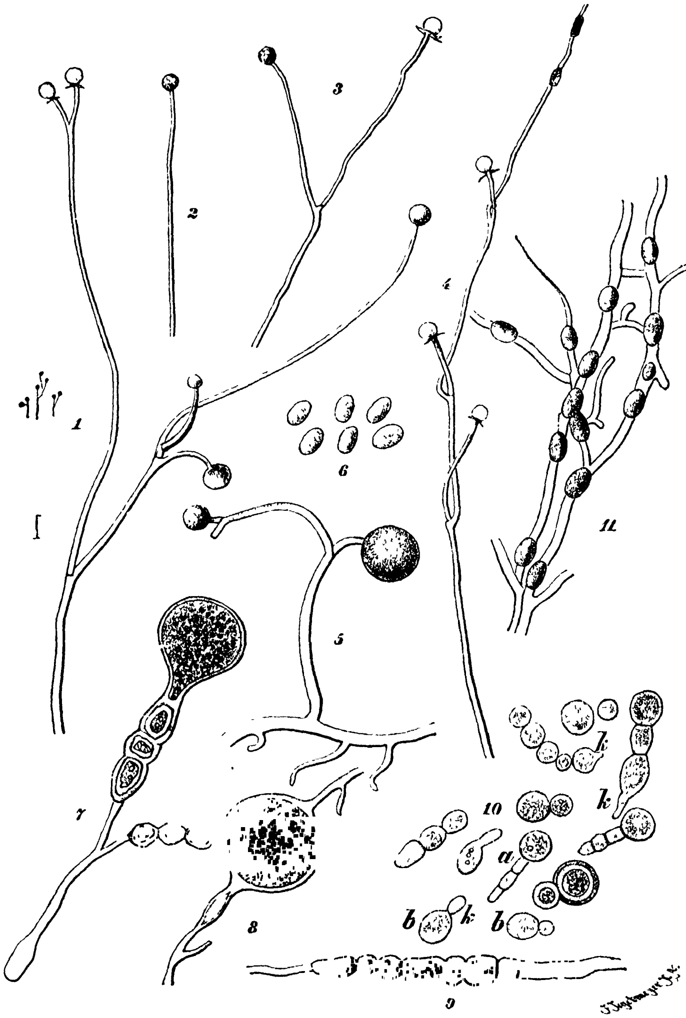


FIG. 64.—*Mucor rouxii.* 1–5, aerial hyphae with sporangia; 6, spores; 7–9, chlamydospores; 10, spherical cells, *a*, germinating, *b*, budding; 11, chlamydospores. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

arises from the matured zygospore, which develops a sporangium at the apex (Fig. 59).

Separation of the various species is based on the length and diameter of the sporangiophores, the type of branching, if any, the size and color of the sporangia, the character of the sporangial wall, zygospores, and

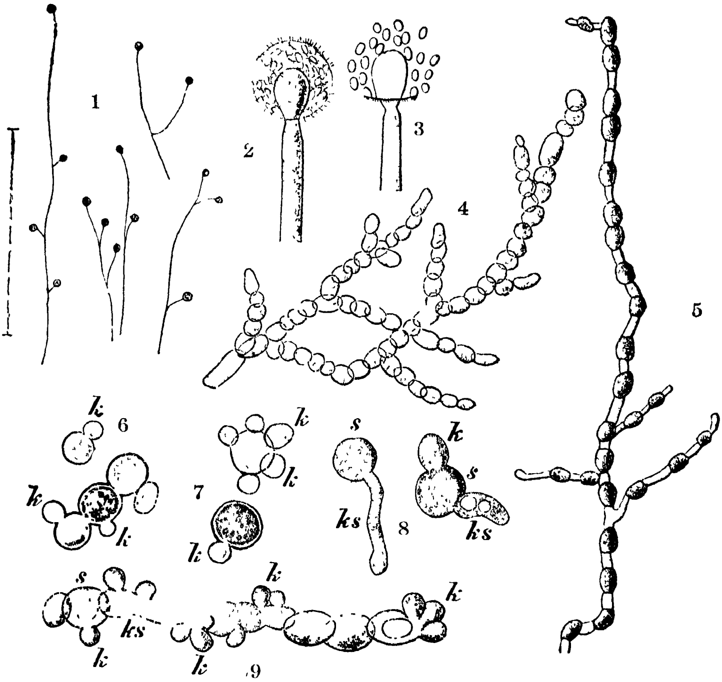


FIG. 65.—*Mucor racemosus.* 1, aerial hyphae with sporangia; 2, sporangium; 3, columella; 4, spherical cells; 5, chlamydospores; 6–7, germination of spherical cells by budding (*k*); 8, germination of a spore; 9 degenerate hypha. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

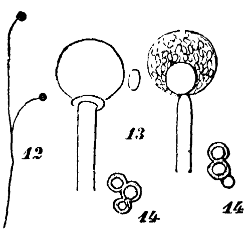


FIG. 66.—*Mucor erectus.* 12, aerial hypha with sporangia; 13, columella (left) and sporangium (right); 14, spherical cells. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

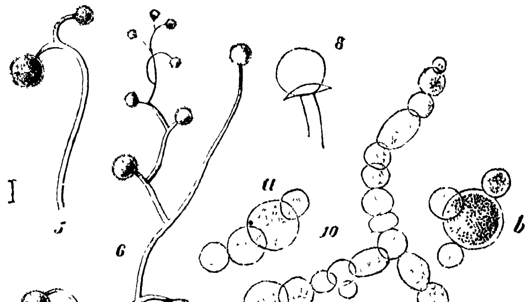


FIG. 67.—*Mucor circinelloides.* 5, 6, 7, sporangiophores and sporangia; 8, columella; 9, spores; 10, spherical cells; 10*b*, germinating spherical cell. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

chlamydospores, the size and shape of the columellae and spores, the colonial characteristics and color, the height of aerial growth, etc.

Rhizopus.—Members of this genus are of common occurrence and frequently cause laboratory contaminations. Growth on the usual

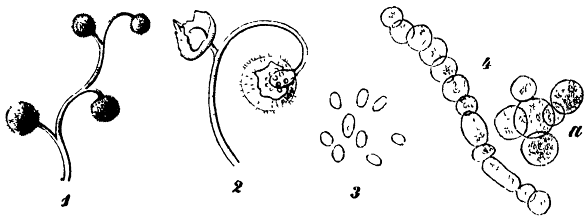


FIG. 68.—*Mucor alternans.* 1, 2, sporangiophores and sporangia; 3, spores; 4, spherical cells; *a*, germinating spherical cell. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

laboratory media is very rapid. The organisms spreaa widely by means of stolons or runners. Culture tubes and Petri dishes soon become filled with a dense cottony mycelium. Species in this genus are easily

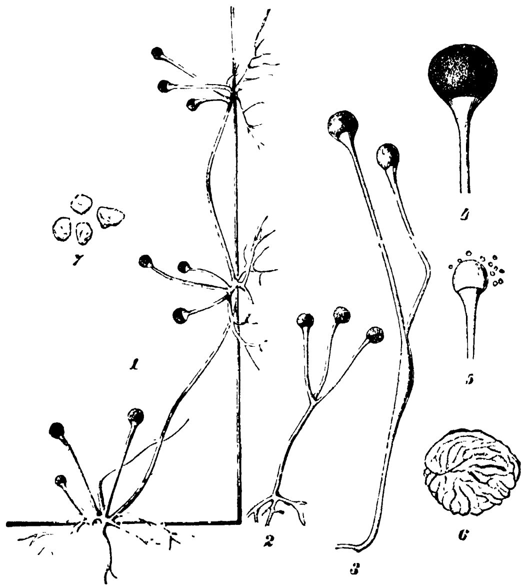


FIG. 69.—*Rhizopus nigricans.* 1, 2, 3, growth showing aerial hyphae and sporangia; 4, sporangium; 5, columella; 6, spore showing wrinkled surface; 7, spores under low power. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

distinguished from members of the *Mucor* by the presence of stolons. Stolons often reach a length of several centimeters and bear tufts of root-like hyphae known as rhizoids, which emerge from the points where the stolons come in contact with the medium or the surface of the glass.

The species are usually grayer in color and produce a more luxurious growth than the mucors (Figs. 69 and 70).

Trichothecium.—This genus contains several species but only one, *T. roseum,* is of common occurrence. The colonies are thin, spreading, floccose, at first white in color, then becoming slowly pale pink. The conidiophores bear clusters of spores attached to the tip. The spores are oval with a nipple-like projection at the point of attachment and are composed of two cells.

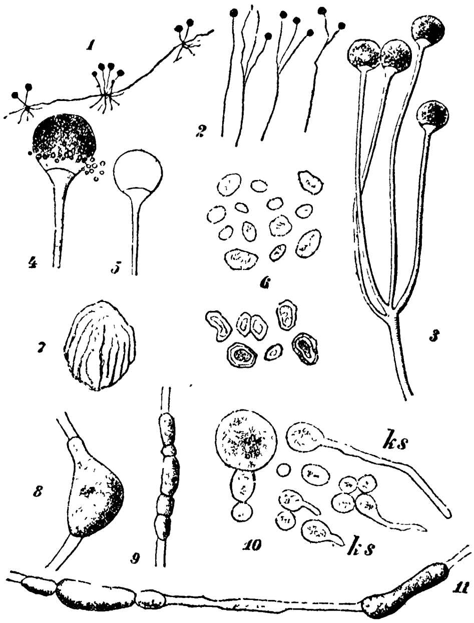


FIG. 70.—*Rhizopus oryzae.* 1, 2, 3, aerial hyphae and sporangia; 4, sporangium; 5, columella; 6, spores under low power; 7, spore under high power; 8, 9, 11, chlamydospores; 10, germination of spherical cells. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

Oidium and Oospora.—There appears to be some doubt as to the correct use of these two terms. Some authorities use the generic name *Oidium* for the parasitic members and the term *Oospora* for the saprophytic species. Others classify under *Oidium* those species having rectangular mycelial fragments and as *Oospora* those species having rounded cells.

A well-known saprophytic species is *Oospora lactis.* It is found in the various kinds of milk products. It grows readily on milk or wort agar and produces a thin, spreading, slimy growth. The colonies are creamy white in color. Young cultures show long hyphal threads while old cultures are composed entirely of short rectangular fragments. Each fragment is capable of producing a new culture (Fig. 71).

Another species, *Oospora crustacea* is found on cheese rind and produces an orange-colored pigment. It produces a powdery type of growth. The optimum temperature is about 18°C. It grows rapidly on culture media and produces a bright orange to scarlet pigment. The cells composing a young mycelium round up before breaking apart and resemble a chain of conidia.

Monilia.—This genus contains only one species of common occurrence. *Mo. sitophila* is found in laboratory air and causes contaminations of

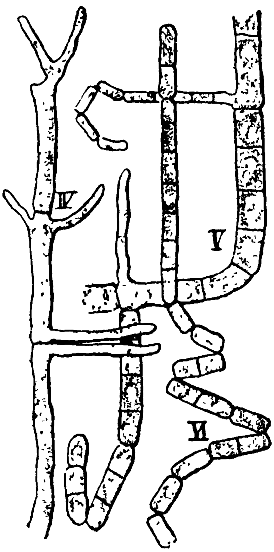


FIG. 71.—*Oospora* (Oidium) *lactis*. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

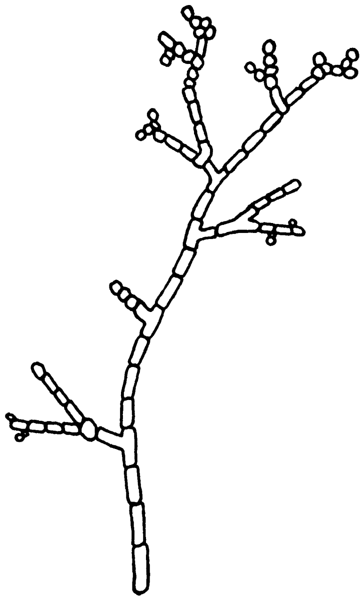


FIG. 72.—*Monilia sitophila*. Aerial hyphae (conidiophores) with budding yeast conidia. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

cultures and media. It grows rapidly on media in loosely floccose masses and produces a pale pink to salmon pink color. The aerial hyphae contain oval-shaped conidia at their tips. The conidia increase by budding, eventually producing large, irregular masses. As the hyphae age, the individual cells break apart. Each cell is capable of producing a new plant (Fig. 72).

Aspergillus.—The species of this genus are relatively common in air. They are found almost everywhere on nearly all types of substrates (Figs. 73, 74, 75, 76, 77, 78, 79). The organisms are found on decaying fruits, vegetables, grains, bread, and other articles of food. Aspergilli are commonly found in incompletely sterilized culture media. The color

may vary considerably. It may appear green, yellow, orange, black, or brown. The molds have a powdery appearance. In marked contrast to *Mucor* the hyphae are branched and septate. The hyphae enlarge at the apex to form conidiophores. The conidiophores are not branched. Numerous short stalks called sterigmata (singular, sterigma) develop from the apical or swollen ends of the conidiophores. Chains of spores are produced from the tips of the sterigmata, sometimes developing to a

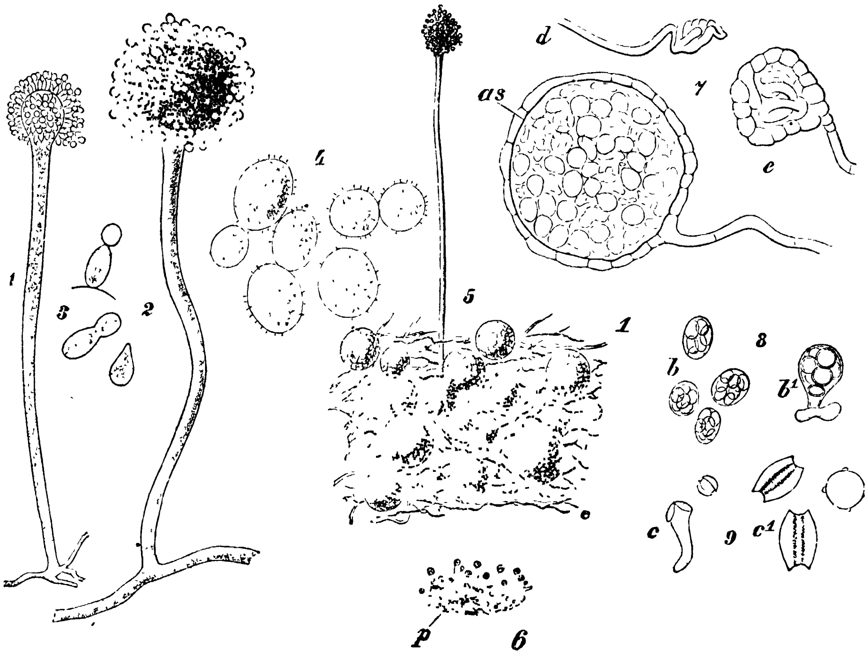


FIG. 73.—*Aspergillus glaucus.* 1–2, conidiophores; 3, sterigmata; 4, conidia; 5, a portion of mycelium with perithecia and conidiophore; 6, same, natural size; 7, sections of perithecia; 8, isolated asci; 9, spores. (*After deBary and Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

considerable length (Fig. 80). A few species produce perithecia (singular, perithecium). These are spherical, cylindrical, or flask-shaped, hollow structures, which contain the asci and usually open by a terminal pore. The asci contain the ascospores. For an excellent classification of the species of *Aspergillus* see the monograph by Thom and Church (1926).

Penicillium.—The members of this genus are closely related to the aspergilli and are also widely distributed in nature. The genus includes the characteristic blue-green colored mold so often observed on citrus and other fruits, vegetables, grains, hay, organic infusions, cheeses, and other food materials (Figs. 81, 82, 83). The vegetative mycelium penetrates the food substances, after which aerial hyphae or conidiophores appear. The conidiophores branch one or more times from the same

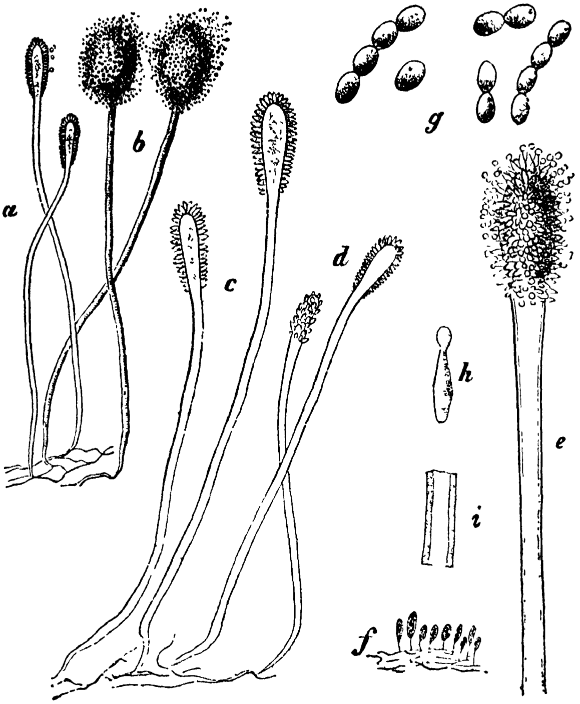


FIG. 74.—*Aspergillus clavatus.* *a, b, c, d, e,* conidiophores in various stages of development; *f,* conidial herbage; *g,* conidia; *h,* sterigma; *i,* section of stem. (*After Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

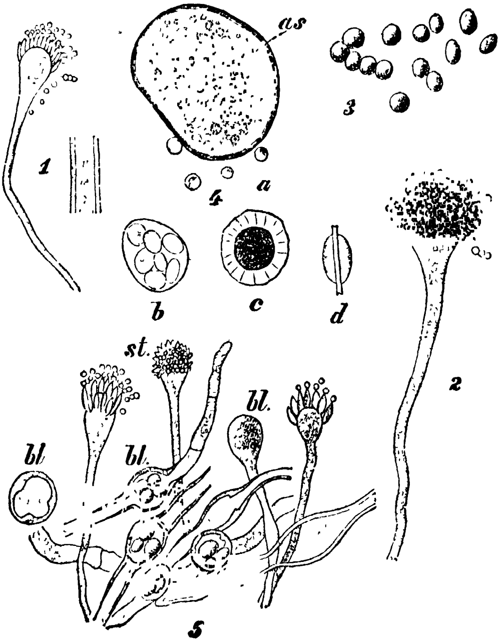


FIG. 75.—*Aspergillus fumigatus.* 1–2, club-shaped conidiophores; 3, conidia; 4, ascus and spores; 5, hyphae with globular swellings. (*After Grijns and Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

joint, giving rise to a terminal cluster of parallel hyphae. The small terminal branches are known as sterigmata. A chain of conidia or spores develop from each sterigma (Fig. 84).

Some species are destructive and others are beneficial. The most important species are *P. roqueforti* and *P. camemberti*, which are responsible for the desirable changes occurring in Roquefort, Camembert,

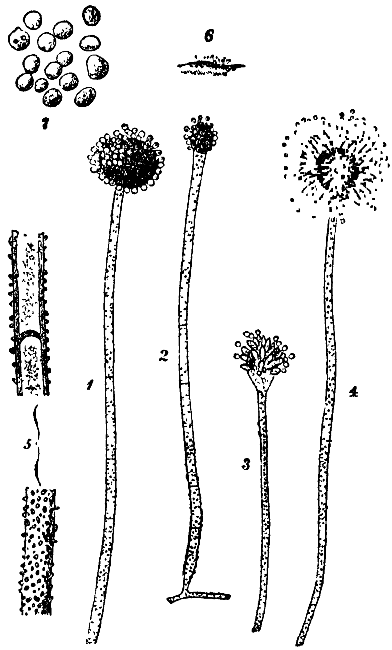


FIG. 76.—*Aspergillus flavus.* 1–4, conidiophores with spherical to club-shaped globules and simple sterigmata; 5, colorless granules on outer wall of stem; 6, conidial herbage; 7, conidia. (*After Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

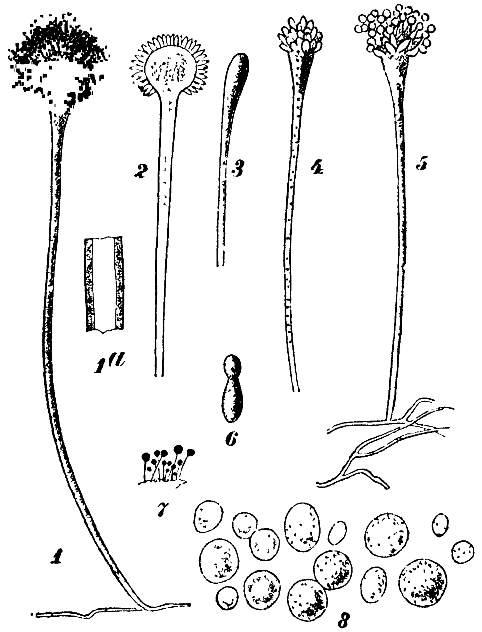


FIG. 77.—*Aspergillus oryzae.* 1, conidiophore; 2, longitudinal section; 3–5, development of conidiophore; 1*a*, longitudinal section of stem; 6, sterigma; 7, conidial herbage; 8, conidia. (*After Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

Gorgonzola, and other cheeses. The penicillia are employed in the manufacture of a considerable number of substances of commercial importance. These are discussed under the Biochemistry of the Molds (page 109).

The species of *Penicillium* are more difficult to classify than are the members of the genus *Aspergillus*. The conidia show less variation in color. Most species show some shade of green during the period of active growth. The colors vary in shade under different environmental conditions and with the age of the cultures. For excellent discussions

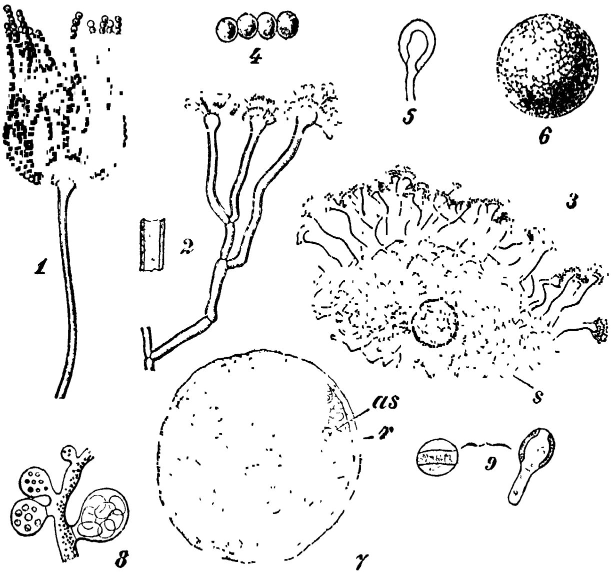


FIG. 78.—*Aspergillus nidulans.* 1, 2, conidiophores with branched sterigmata; 4, conidia; 5, 6, 7, ascospore with globular capsule; 8, asci; 9, spores. (*After Eidam; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer Jena.*)

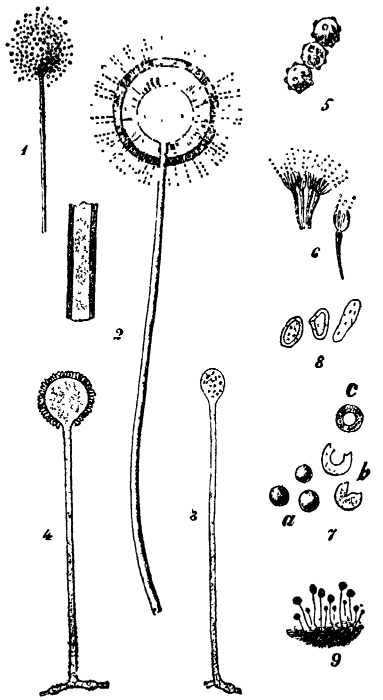


FIG. 79.—*Aspergillus niger.* 1, 2, conidiophores; 3, young conidiophore before formation of sterigmata; 4 young conidiophore during formation of sterigmata; 5, globular, warty conidia; 6, sterigmata; 7, sclerotia; 8, tough skinned spotted cells from interior of sclerotia; 9, conidial herbage. (*After Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

of the penicillia see the monographs by Smith (1938) and by Thom (1930).

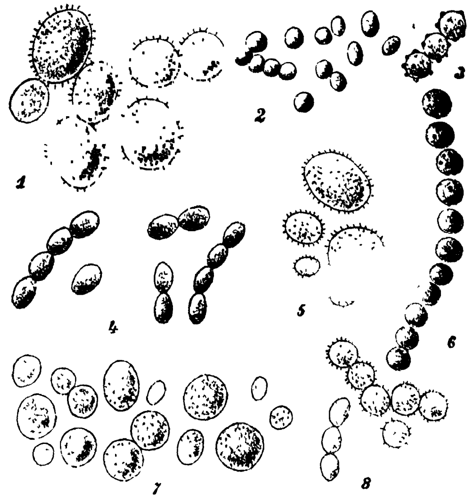


FIG. 80.—Conidia of various species of *Aspergillus*. 1, *A. glaucus;* 2, *A. fumigatus;* 3, *A. niger;* 4, *A. clavatus;* 5 *A. tokelau;* 6 *A. varians;* 7, *A. oryzae;* 8 *A. wenti*. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

Cladosporium.—The most common species in this genus is *C. herbarum*. It is widely distributed, being found on rubber, leather, textiles,

FIG. 81.—*Penicillium brevicaule*. 1, 2, formation of conidia on special conidiophores, 3, formation of conidia on the mycelium. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

foodstuffs, and decaying vegetable matter (Fig. 85). On culture media it produces a thick, velvety growth and the color varies from deep green

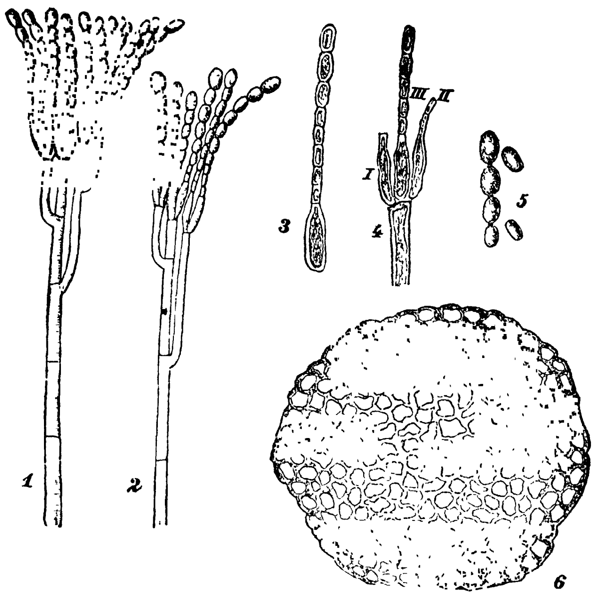


FIG. 82.—*Penicillium italicum.* 1, 2, conidiophores; 3, 4, sterigmata; 5, conidia; 6, section through an old sclerotium. (*After Wehmer; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

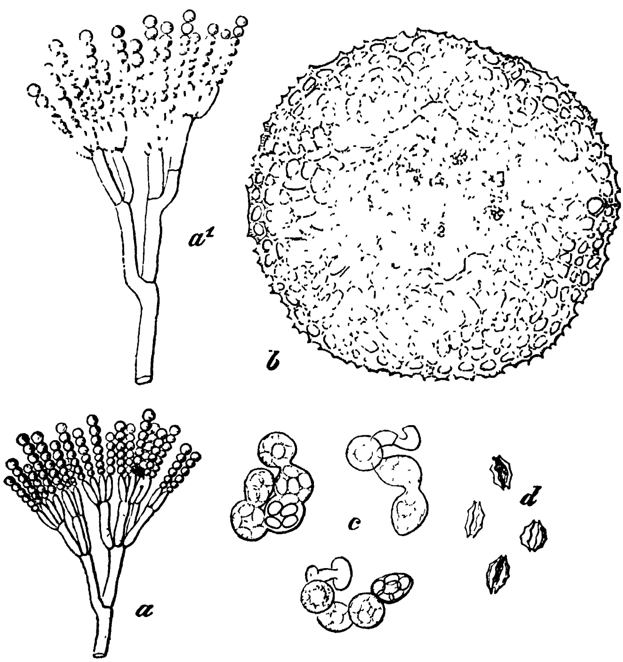


FIG. 83.—*Penicillium glaucum.* *a*, a^1, conidiophores showing branching; *b*, perithecium with ripening asci; *c*, ascus showing germination; *d*, spores, viewed laterally. (*After Brefeld; from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

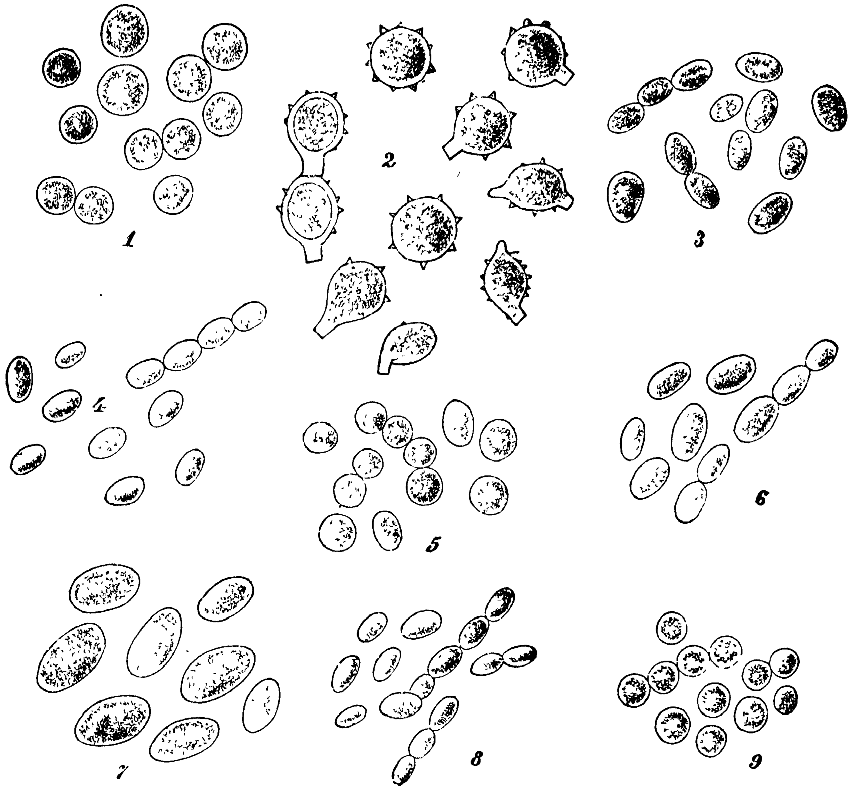


FIG. 84.—Conidia of various species of *Penicillium*. 1, *P. camemberti;* 2, *P. brevicaule;* 3, *P. purpurogenum;* 4, *P. claviforme;* 5, *P. rubrum;* 6, *P. italicum;* 7, *P. olivaceum;* 8, *P. luteum;* 9, *P. glaucum.* (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

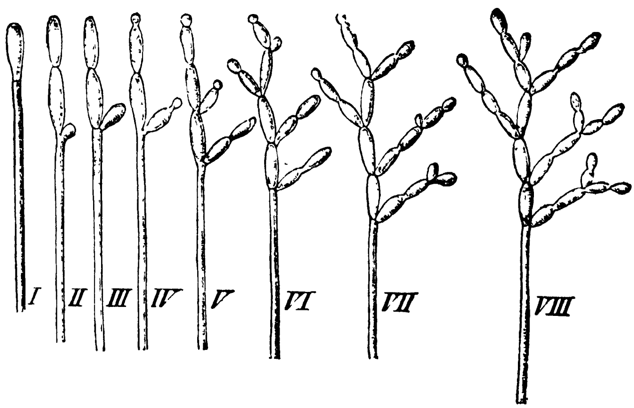


FIG. 85.—*Cladosporium herbarum.* Successive steps in the development of conidia. (*From Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

to dark gray-green. When examined microscopically in the dry state the spores occur in large, almost tree-like clusters. In mounted specimens the structure breaks up, the spores detach themselves, and the hyphae separate into rod-like cells. The oval-shaped spores increase by budding in a manner similar to the yeasts. The young spores are usually single-celled; the old spores frequently show two cells.

Alternaria.—The species are commonly found on organic compounds. Parasitic forms have been isolated from cultivated plants. On culture media the organisms grow rapidly and produce dense, floccose, greenish-colored mycelium. The mycelium is septate and may form chains of short, swollen cells similar to oidiospores. The conidia vary from oval-shaped to roughly club-shaped forms, with a pronounced beak at the tip. The spores are produced in short chains, sometimes branched, and are greenish brown to dark brown in color. They show both transverse and longitudinal septa and the degree of division increases with age.

LABORATORY TECHNIQUE

In order that accurate studies be made on molds it is necessary to grow them in pure cultures. The methods employed for isolating and studying the growth of molds in pure cultures are, in general, similar to those used for bacteria (page 116). Most molds grow best at a temperature of about 25°C.

Culture Media.—Many types of solid and liquid culture media are employed for the cultivation of molds but only a few of these are for general use. Media employed for the cultivation of molds are usually slightly acid in reaction. Many species are able to tolerate relatively high acidities. Vegetables and vegetable extracts are commonly incorporated in culture media. The solid media may be composed of solid substances such as potatoes, carrots, and beans, or liquid media made solid by the addition of agar or gelatin.

One of the simplest media employed for the cultivation of molds is Czapek's solution. This is not only easy to prepare but is probably as good as any other for general use. A modification of the original formula is as follows:

Sodium nitrate	2.0	gm.
Potassium chloride	0.5	gm.
Magnesium sulfate	0.5	gm.
Ferrous sulfate	0.01	gm.
Potassium acid phosphate	1.0	gm.
Distilled water, to make	1000	cc.

Various carbohydrates and other carbon compounds may be incorporated. Usually 50 gm. of glucose or 30 gm. of sucrose is added. The medium is acid (pH4.2), which is more favorable to mold growth than a neutral reaction. The medium may be solidified by the addition of 15 to 20 gm. of agar per liter. This is probably the most useful solid medium employed for the cultivation of molds.

Microscopical Methods.—Considerable information can be obtained by examining first dry, living cultures under the low-power objective. Petri dish cultures are placed on the stage of the microscope, with the lids removed, and examined by transmitted or reflected light. Aerial mycelium, conidiophores, fruiting heads, chains of

spores, and other structures may be easily examined by this method. This gives a preliminary idea of what to look for when slide preparations are examined, since mold structures are easily broken when disturbed.

For high-power examination, slide preparations are necessary. Mold specimens are very difficult to remove from culture media without being greatly broken. Therefore, great care must be exercised in preparing satisfactory mounts. Water should not be used for the mounting fluid since it rapidly evaporates, produces a shrinkage of the hyphae by osmosis, and causes the various parts to adhere together as a tangled mass. Obviously such preparations are unsatisfactory for accurate observations. Probably the most useful mounting medium is that known as lactophenol. It has the following composition:

Phenol, c.p. crystals	20 gm.
Lactic acid, c.p.	20 cc.
Glycerin, c.p	40 cc.
Distilled water	40 cc.

The phenol is first dissolved in the water, then follows the addition of the other constituents.

This fluid does not cause shrinkage of the cells and does not evaporate, thus permitting permanent preparations to be prepared. A dye may be added to the fluid to stain the various mold structures. This is especially desirable for mounting molds that are to be photographed.

Molds are mounted by first placing a drop of lactophenol in the center of a clean glass slide. A small portion of the mold growth is removed from the culture and placed in the drop of fluid. It is gently teased out with a pair of needles until the various parts are well separated and wetted by the fluid. It is then carefully covered with a cover slip to avoid, as far as possible, air bubbles being entrapped. For an excellent discussion of mycological methods see the monograph by Smith (1938).

BIOCHEMISTRY OF THE MOLDS

The biochemical activities of molds are of great importance in the industrial world. They are probably not so important in this respect as are the yeasts and bacteria but they do, nevertheless, produce certain changes not carried on by the other two members of the fungi group.

A few of the more important biochemical changes induced by the growth of molds are the following:

Alcoholic Fermentation.—Alcohol is produced industrially by the fermentation of various sugars by yeasts. The raw materials consist of various sugars such as cane sugar, molasses, and glucose, and the polysaccharides such as potato starch, cornstarch, barley starch, or cellulose.

As a preliminary to their utilization by yeasts, starches and other polysaccharides must be first hydrolyzed to soluble sugars. Several molds elaborate the necessary enzyme or enzymes that effect the saccharification of the various starches, after which certain yeasts are capable of fermenting the sugars to alcohol. Yeasts do not produce an amylase and are unable to ferment starches directly to alcohol.

Fitz (1873) was probably the first to show that the mold *Mucor mucedo* (now recognized as *M. racemosus*) was capable of elaborating the starch-hydrolyzing enzyme amylase or diastase. Since that time many other molds have been shown to be capable of elaborating an amylase. The commercial product Takadiastase, which was first prepared by Takamine (1914) from some species of *Aspergillus*, is extensively used for the saccharification of starches.

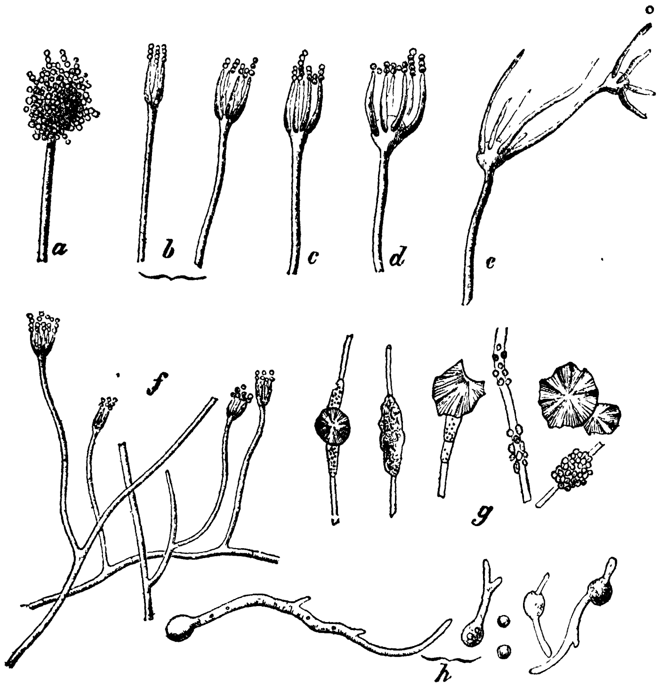


FIG. 86.—*Citromyces (Penicillium) pfefferianus.* *a*, conidiophore; *b–e*, conidiophores after removal of the conidia; *f*, conidiophores slightly magnified; *g*, hyphae, showing spherical, granular, or compact deposits of calcium citrate; *h*, germinating conidia. (*After Wehmer, from Lafar, Handbuch der technischen Mykologie, courtesy of Gustav Fischer, Jena.*)

Citric Acid.—Wehmer (1893) showed that two species of Citromyces (now *Penicillium*) were capable of fermenting sugar with the production of citric acid (Fig. 86). It was shown subsequently that many species of *Penicillium* are also able to produce citric acid in varying amounts but in no instance is the yield sufficiently large to enable the method to compete with the extraction of citric acid from lemons and other citrus fruits. Currie (1917) found that the yield could be greatly increased by employing the mold *Aspergillus niger.* The mold is employed commercially for this purpose and in some localities it is as cheap to prepare citric acid by this method as it is from citrus fruits.

The carbohydrates that have been found suitable for citric acid fermentation include starch, sucrose, glucose, fructose, lactose, invert sugar (glucose + levulose), maltose, molasses, and the higher alcohol, glycerol. The yield of citric acid varies considerably, depending upon the carbohydrate employed. Sucrose gives as high as 15 per cent citric acid.

Currie (1917) found the following medium to yield relatively large quantities of citric acid:

Sucrose	125	gm.
Ammonium carbonate	2	gm.
Potassium acid phosphate	1	gm.
Magnesium sulfate	0.2	gm.
Distilled water	1000	cc.

The fermentation yields oxalic acid and carbon dioxide besides citric acid. The oxalic acid is separated from the fermented mixture by evaporation and crystallization, leaving the citric acid in solution.

The reaction for the formation of citric acid from glucose is as follows:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} + 3O \rightarrow \underset{\text{Citric acid}}{\begin{array}{l} CH_2\cdot COOH \\ | \\ COH\cdot COOH \\ | \\ CH_2\cdot COOH \end{array}} + 2H_2O$$

The intermediate steps are not clearly understood.

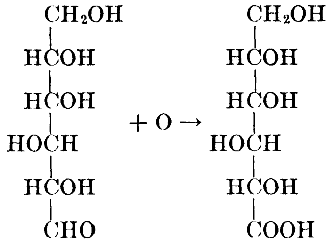
Gluconic Acid.—Molliard (1922) was the first to detect the presence of gluconic acid as one of the products in the fermentation of glucose by the mold *Sterigmatocystis niger*. Later Butkewitsch (1923) identified the same acid in cultures of *Aspergillus niger*. The acid is produced by a number of aspergilli and penicillia. The mold that is usually preferred for this purpose is *Penicillium purpurogenum* var. *rubrisclerotium*.

Chemical methods for the preparation of the acid have not proved successful. It is more easily produced by the fermentation of glucose. Herrick and May (1928) obtained best results from the following medium:

Glucose	200	gm.
Magnesium sulfate	0.25	gm.
Disodium phosphate	0.10	gm.
Potassium chloride	0.05	gm.
Sodium nitrate	1.00	gm.
Distilled water	1000	cc.

The medium produces a good yield in about 10 days when incubated at a temperature of 25 to 30°C.

The reaction for the oxidation of glucose to gluconic acid is as follows:



d-Glucose *d*-Gluconic acid

Cheeses.—Many cheeses are ripened by means of molds. These may be placed in two groups: (1) the soft cheeses of the Camembert and Brie types and (2) the green-streaked cheeses of the Roquefort, Gorgonzola, and Stilton types.

The cheeses in the first group are ripened by means of the mold *Penicillium camemberti*. The prepared curd is shaped into cakes, salted on the surface, and inoculated with the spores of the mold. The cakes are placed in a damp room where the mold rapidly multiplies on the surface, then gradually softens the entire mass of curd. The process requires about four weeks.

The cheeses in the second group are prepared by first inoculating the curd with a pure culture of *P. roqueforti*. The curd is then pressed so as to leave irregular cracks in the cake. The cake is aerated from time to time during the ripening process by piercing it with wires. The mold produces a dense growth along the cracks, giving the finished product a streaked appearance.

Miscellaneous Compounds.—Molds produce a large number of compounds that are not of commercial importance at present. They are nonnitrogenous, metabolic products and are probably the result of the action of the organisms on carbohydrates or carbohydrate-like compounds. It should be noted that, on the whole, molds produce compounds of greater complexity than do bacteria.

Most of the compounds that have been isolated and characterized are the following:

Acids.—Aconitic, carlic, carlosic, carolic, carolinic, 3:5-dihydroxyphthalic, dimethylpyruvic, fulvic, fumaric, gallic, gentisic, glycuronic, glycolic, glyoxylic, 2-hydroxymethylfurane-5-carboxylic, itaconic, kojic, γ-ketopentadecoic, lactic, malic, *d*-mannonic, 1-γ-methyltetronic, 6-hydroxy-2-methylbenzoic, minioluteic, mycophenolic, oxalic, penicillic, puberulic, pyruvic, spiculisporic, succinic, and terrestric.

Pigments.—Aurantin, aurofusarin, auroglaucin, β-carotene, boletol, catenarin, chrysogenin, citrinin, citromycetin, cynodontin, erythroglau-

cin, flavoglaucin, fulvic acid, helminthosporin, monascoflavin, monascorubrin, oosporin, physcion, ravenelin, and tritisporin.

Polysaccharides.—Capreolinose, galactocarolose, glycogen, luteic acid, mannocarolose, mold starch, mycodextrin, rugulose, and varianose.

Miscellaneous.—Mannitol, glycerol, fumigatin, mellein, spinulosin, and terrein.

For an excellent discussion of the subject consult the monographs by Raistrick (1932, 1938).

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CHAPTER VII

TECHNIQUE OF PURE CULTURES

GENERAL CONSIDERATIONS

A culture may be defined as the active growth of microorganisms in or on nutrient media.

A mixed culture consists of two or more species of organisms growing together.

A pure culture consists of only one species of microorganism growing in or on nutrient media. Pure cultures are required for studying the morphology and physiology of organisms. All laboratory studies, with very few exceptions, are based on the use of pure cultures. In a few instances two species are grown together in making a study of the various types of bacterial associations.

Plate cultures are cultures of organisms grown on a solid medium contained in Petri dishes.

Slant cultures are those grown on the inclined surface of solid media, such as nutrient agar, coagulated blood serum, or potato. These are referred to specifically as nutrient agar slant cultures, blood serum slant cultures, potato slant cultures, etc., respectively. Cultures prepared in this manner are sometimes referred to as streak cultures. A nutrient agar slant culture may, for example, be called a nutrient agar streak culture. Solid media prepared in the slanted position greatly increase the surface area exposed to the air, resulting in a much greater growth of organisms.

A stab culture is one prepared by stabbing a solid medium, such as nutrient agar or nutrient gelatin, to a considerable depth with a previously inoculated straight wire needle. Gelatin medium is used for studying the character of liquefaction produced by certain organisms. If a fermentable carbohydrate agar medium is used, the production of gas may be detected by the separation of the agar into several layers.

Liquid cultures are those prepared by inoculating the organisms into liquid media, such as nutrient broth, milk, or Dunham's peptone solution.

Shake cultures are those prepared by inoculating liquefied agar media and rotating or shaking the tubes to obtain a uniform suspension of the organisms before solidification takes place. A shake culture is valuable for indicating the oxygen requirements of an organism. The anaerobic

organisms grow in deeper portions of the medium; the aerobes grow near the surface in the presence of a plentiful supply of oxygen.

METHODS EMPLOYED FOR THE INOCULATION OF CULTURE MEDIA

Agar Deep Cultures.—Sterilize a wire needle in a flame and allow it to cool for a few moments. Remove the cotton stopper from an agar slant culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Hold the tube slanted, not upright, to minimize aerial contamination. Remove a small amount of the growth with the sterilized wire needle. Again flame the neck of the agar slant culture, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from the tube to be inoculated by grasping it with the small finger of the right hand. Flame the neck of the tube. Stab the straight wire containing the inoculum to the bottom of the tube. Withdraw the needle carefully. Again flame the neck of the tube and replace the cotton stopper. Flame the wire needle before setting it down on the table. Incubate the culture at the proper temperature.

If a transfer is to be made from a liquid culture, use a wire loop instead of a needle. Remove a loopful of the medium and force the wire loop to the bottom of the tube. Withdraw the loop carefully. The procedure in every other detail is the same as above.

Agar Slant Cultures.—Sterilize a wire needle or wire loop in the flame, depending upon whether a solid or a liquid culture is to be used. Allow the wire to cool for a few moments. Remove the cotton stopper from the culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Remove a small amount of the growth with the sterilized wire needle, or a loopful of the liquid culture with the wire loop. Again flame the neck of the culture, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from the agar slant to be inoculated by grasping it with the small finger of the right hand. Flame the neck of the tube. Spread the inoculum over the surface of the agar slant by making streaks back and forth a few millimeters apart. Start at the butt of the slant and work up to the top. Withdraw the needle or loop from the tube. Again flame the neck of the tube and replace the cotton stopper. Flame the wire needle or loop before setting it down on the table. Incubate the culture in an upright position at the proper temperature.

Broth Cultures.—Follow the same procedure as used for the preparation of agar slant cultures except that the inoculating needle or loop is plunged into broth and shaken to dislodge the inoculum from the wire.

Isolation of Species.—Bacteria are rarely found in nature as single species. Mixed species is the rule. An organism must first be isolated

and grown in pure culture before it can be studied accurately. Two different species growing together may produce reactions quite different from those given by each organism when studied separately.

Plate cultures offer a means for isolating pure cultures of organisms in a comparatively simple manner. Two methods are generally followed: (1) the streak plate method and (2) the pour-plate method.

Streak Plate Method.—Melt two tubes of nutrient agar in boiling water or in an Arnold sterilizer. Allow the agar to cool to about 50°C. Remove the cotton stopper from one of the tubes and flame the opening. Lift the lid of a sterile Petri dish just high enough to insert the opening of the test tube and pour the melted agar into the plate. In like manner pour the second tube of agar into another sterile Petri dish. Great care must be observed in pouring agar from a test tube into a Petri dish to avoid external contamination. Always flame the neck of the test tube. Also, never raise the lid of the Petri dish any more than is necessary. Set both plates aside until the agar has become firm.

Sterilize the wire loop in a flame and allow it to cool a few moments. Remove the cotton stopper from a broth culture by grasping it with the small finger of the right hand and flame the neck of the tube. Remove a loopful of the culture with the wire loop. Again flame the neck of the culture tube, replace the cotton stopper, and set the tube in the test-tube block. Raise the lid of the Petri dish high enough to insert the wire loop. Spread the loopful of culture at the upper end of the dish to thin it out; then make streaks back and forth with a free-arm movement from the elbow, over the surface of the agar, about ¼ in. apart. The first streak will contain more of the culture than the second, the second streak more than the third, etc. The last streaks should thin out the culture sufficiently to give isolated colonies. It is usually advisable to streak a second plate, without reinoculationg the wire loop. This gives greater certainty in securing well-isolated colonies. Each colony usually represents the growth from a single organism. Incubate the plates at the proper temperature.

The colonies appear only on the surface of the agar. A pure culture may be obtained from a well-isolated colony by transferring a portion with the wire needle to an appropriate culture medium (Fig. 87).

Pour-plate Method.—Melt three tubes of nutrient agar in boiling water or in an Arnold sterilizer. Allow the agar to cool to about 50°C. Sterilize a wire loop in a flame and allow it to cool for a few moments. Remove the cotton stopper from the culture, by grasping it with the small finger of the right hand, and flame the neck of the tube. Remove a loopful of the liquid culture with the wire loop. Again flame the neck of the culture tube, replace the cotton stopper, and set the tube in the test-tube block. Remove the cotton stopper from one of the tubes of

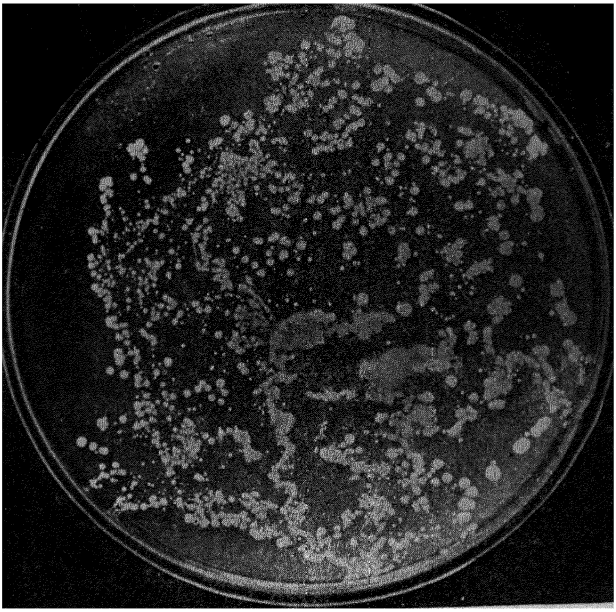


FIG. 87.—Isolation of species by the streak method. The culture contained a mixture of *Escherichia coli* and *Staphylococcus aureus*.

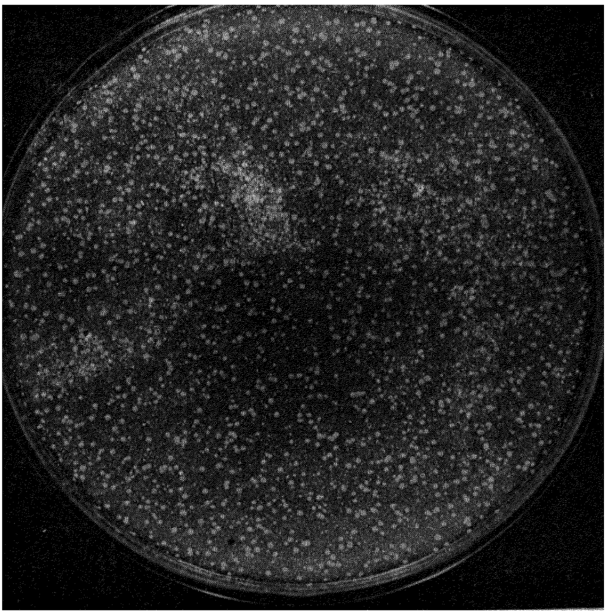


FIG 88*A*.—Isolation of species by the pour-plate method. Agar inoculated with one loopful of a mixture of *Escherichia coli* and *Staphylococcus aureus*.

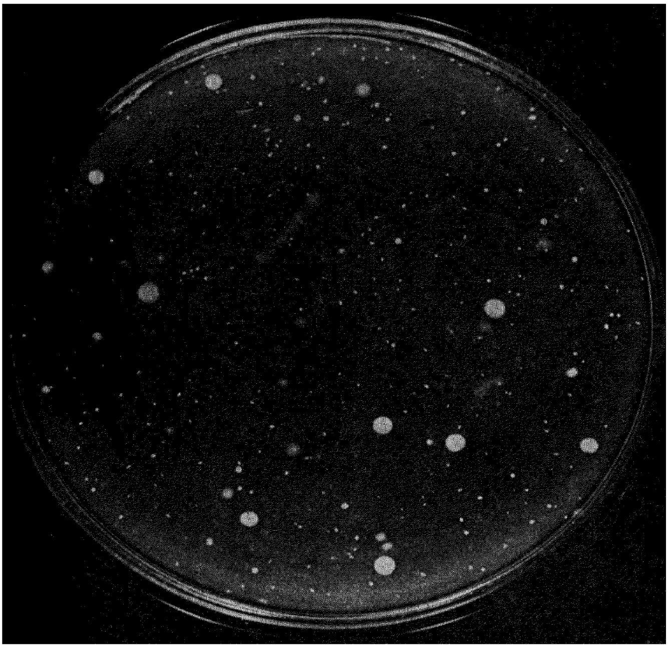


FIG. 88*B*.—Agar inoculated with one loopful from tube shown in Fig. 88*A*.

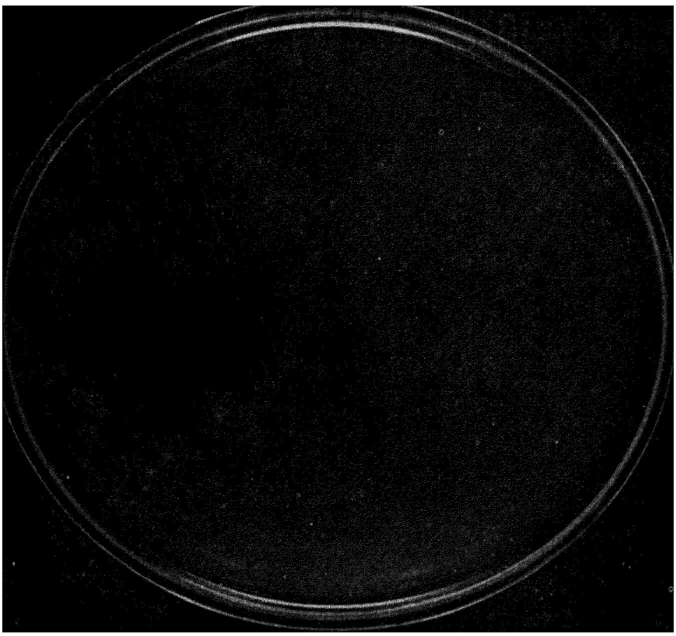


FIG. 88*C*.—Agar inoculated with one loopful from tube shown in Fig. 88*B*.

melted and cooled agar, by grasping it with the small finger of the right hand, and flame the neck of the tube. Plunge the inoculating loop into the agar and shake to remove the inoculum from the wire before withdrawing from the tube. Again flame the neck of the tube and replace the cotton stopper. Flame the wire loop before setting it down on the

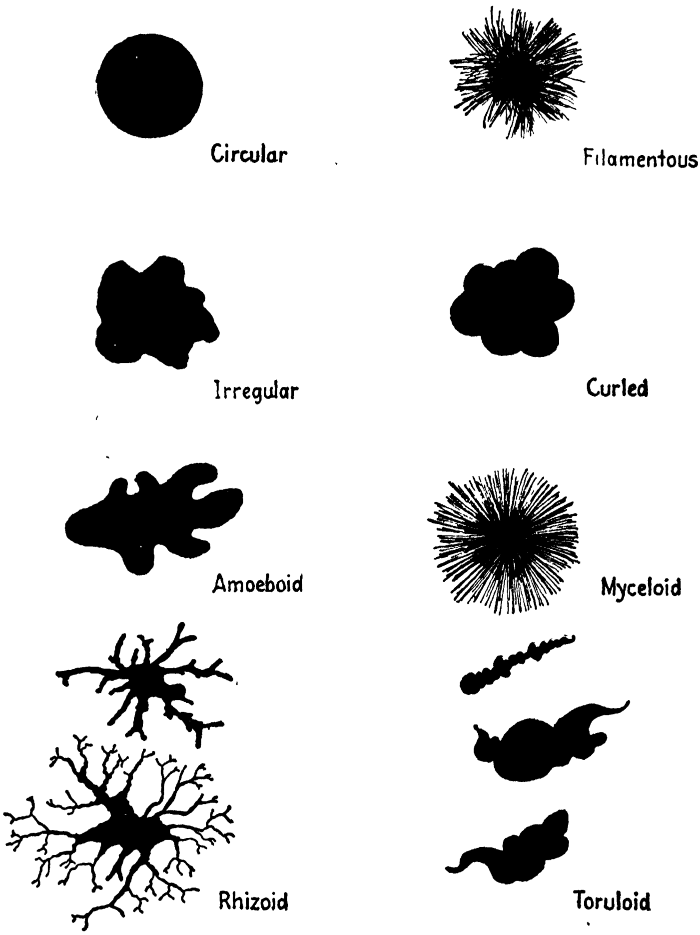


FIG. 89.—Shape or form of colonies. (*After Thomas.*)

table. Mix thoroughly by rotating the tube between the palms of the hands to obtain a uniform suspension of organisms. Remove a loopful from tube 1 and transfer to tube 2. Again rotate the tube between the palms of the hands to mix thoroughly. Remove a loopful from tube 2 and transfer to tube 3. Mix thoroughly as before. Pour the inoculated agar into three sterile Petri dishes. When firm, incubate the plates at the proper temperature (Fig. 88*A*, *B*, *C*).

In this procedure most of the colonies are embedded in the agar and only a few occur on the surface. The first agar plate usually contains too many organisms with the result that it is a difficult matter to remove a well-isolated colony. The colonies are so crowded that they are not able to develop to their normal size. The second or third plate should

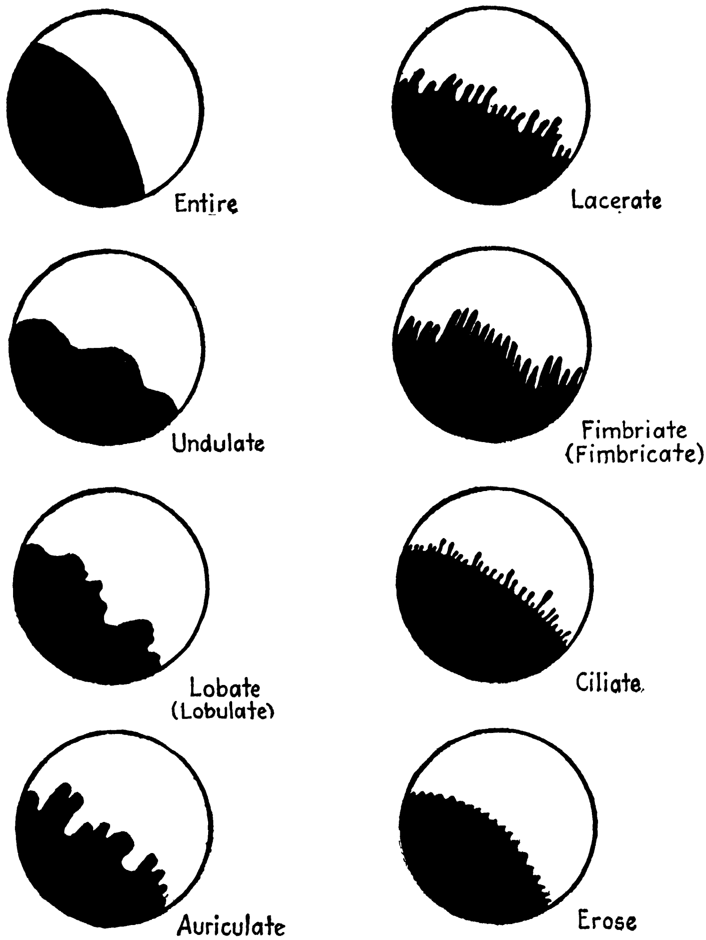


FIG. 90.—Edge of colonies. (*After Thomas.*)

show well-separated colonies of normal size. A pure culture may be obtained by removing a portion of a well-separated colony with the wire needle and transferring it to an appropriate medium.

Well-isolated surface colonies are usually round and quite characteristic for each species. On the other hand, colonies embedded in the agar are smaller in size and usually lenticular in shape. As a rule it is

not possible to distinguish between different species of subsurface colonies by their colonial appearance.

It is usually easier to obtain pure cultures by the pour-plate method because the organisms separate better when mixed with melted agar. Bacteria that produce mucoid colonies are very difficult to separate from nonmucoid organisms by the streak plate method.

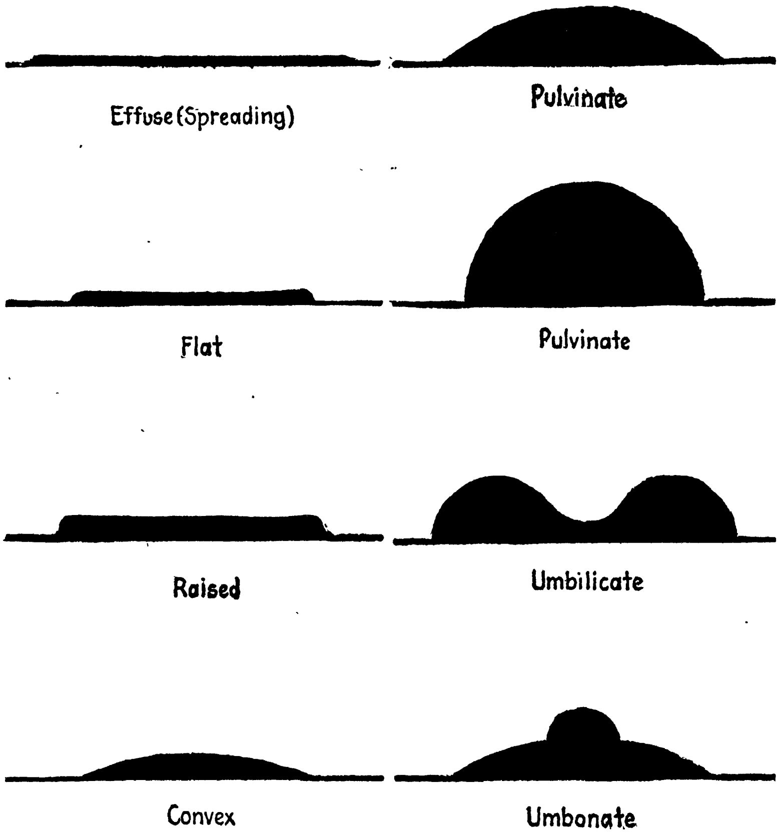


FIG. 91.—Elevation of colonies. (*After Thomas.*)

IDENTIFICATION OF BACTERIAL SPECIES

A descriptive chart, prepared by the Committee on Bacteriological Technic of the Society of American Bacteriologists, will be found on page 124. The chart is used for the identification and classification of bacteria

Colony Formation.—Each bacterial species, when grown on a standard solid medium, forms a characteristic type of colony. Colonies differ in size, shape, edge, elevation, internal structure, etc. The various characteristics are illustrated in Figs. 89, 90, 91 and 92.

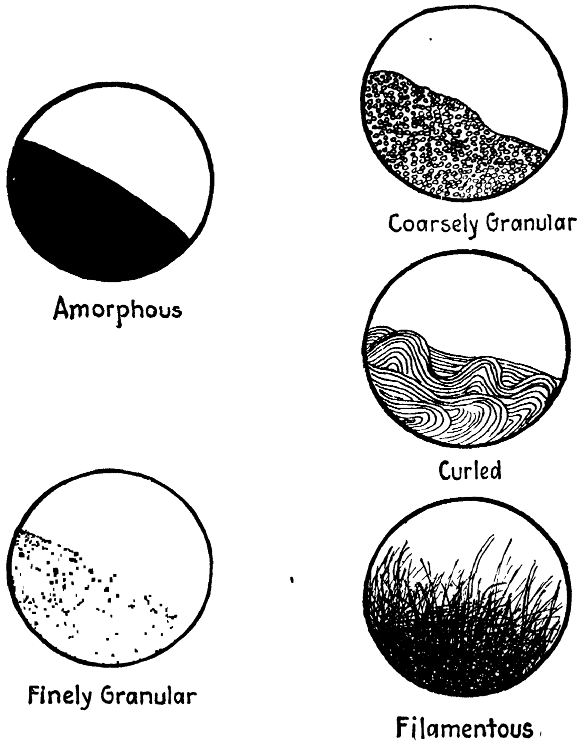


FIG. 92.—Internal structure of colonies. (*After Thomas.*)

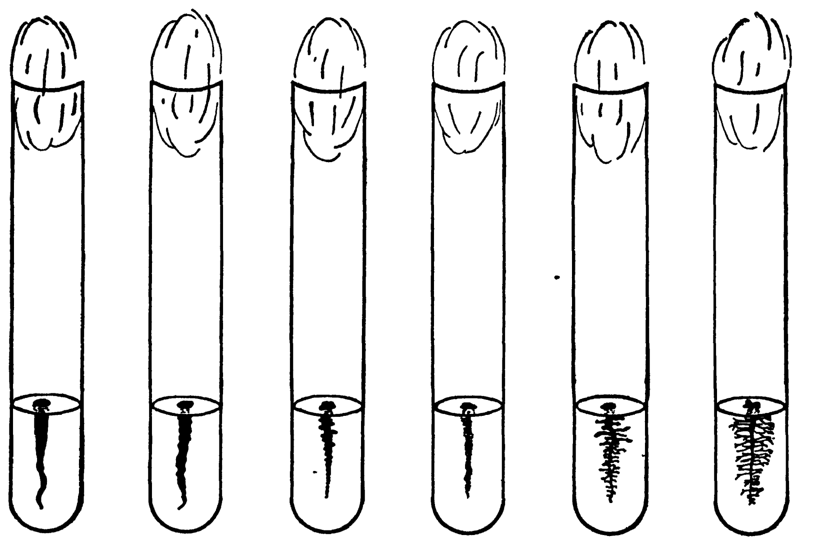


FIG. 93.—Growth in agar stab cultures. (*After Thomas.*)

Stab and Streak Cultures.—The type of growth on the surface and in the depth of agar media is characteristic for many bacterial species

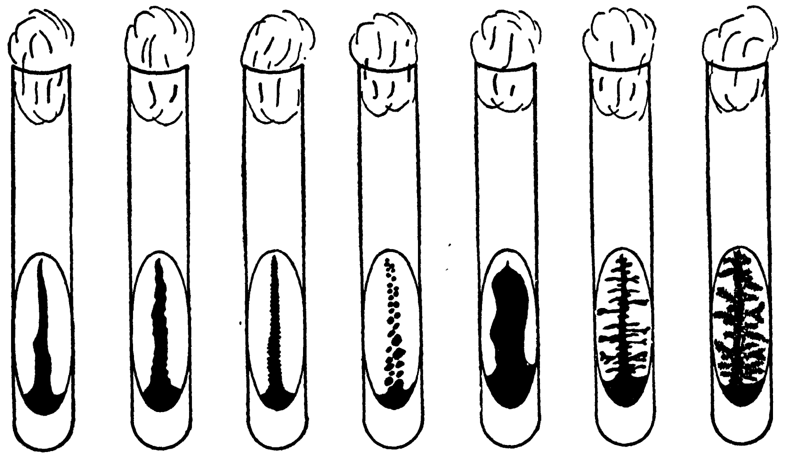


FIG. 94.—Growth on agar streak cultures. (*After Thomas.*)

(Figs. 93 and 94). This applies also to the character of the liquefaction of gelatin stab cultures (Fig. 95).

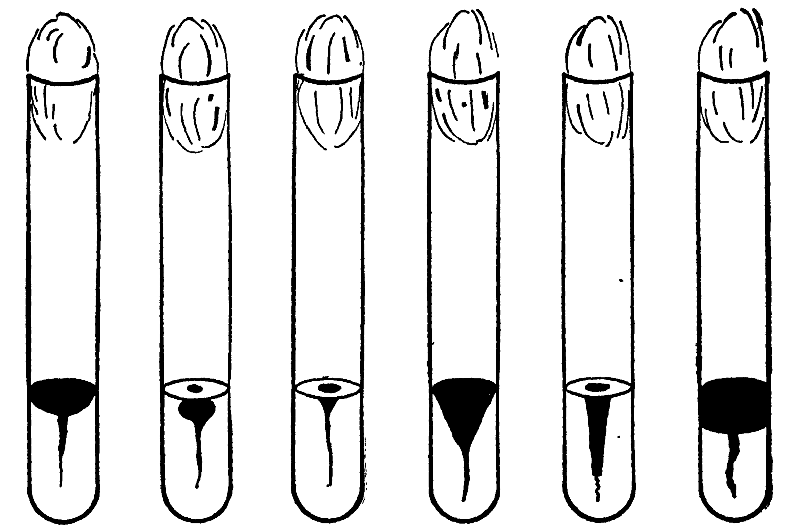


FIG. 95.—Growth in gelatin stab cultures. (*After Thomas.*)

These various characteristics are made use of in the differentiation and classification of bacteria.

Name of organism ______________________ Source ______________________
Date of isolation ______________________ Habitat ______________________
Is phase variation observed? ________ Phase on this Chart: S, R, M, G (smooth, rough, mucoid, gonidial) ________

Underscore required terms.

SKETCHES

VEGETATIVE CELLS: Medium used ______________
Reaction (pH) ______ Temp. ______ Age ____ d.
Size of Majority ______________
Ends, *rounded*, *truncate*, *concave*, *tapering*

MOTILITY: In broth ____________ On agar ____________
[illegible]
[illegible] ...e ____ d.
[illegible]

IRREGULAR FORMS:
Present on __________ in ________ days at ________ °C.

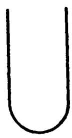
AGAR COLONIES: Temperature ______ °C. Age _____ d.
[illegible] 1 *m.m. diam.*), *circular* (*i.e. over* [illegible] *irregular*, *rhizoid*.
Surface, *smooth*, *rough*, *concentrically ringed*, *radiately ridged*.
Edge, *entire*, *undulate*, *lobate*, *erose*, *filamentous*, *curled*.
Elevation of growth, *effuse*, *flat*, *raised*, *convex*.
Optical Characters, *opaque*, *translucent*, *opalescent*, *iridescent*.

Surface Colonies | Deep Colonies

GELATIN COLONIES: Temperature ______ °C. Age _____ d.
Form, *punctiform*, *circular*, *irregular*, *filamentous*.
Elevation, *flat*, *raised*, *convex*, *pulvinate*, *crateriform* (*liquefying*).
Edge, *entire*, *undulate*, *lobate*, *erose*, *filamentous*, *curled*.
Liquefaction, *cup*, *saucer*, *spreading*.
Surface, *smooth*, *contoured*, *rugose*.
Optical Characters, *opaque*, *translucent*, *opalescent*, *iridescent*.

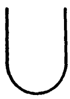
Surface Colonies | Deep Colonies

AGAR STROKE: Temperature ______ °C. Age _____ d.
Growth, *scanty*, *moderate*, *abundant*, *none*.
Form of growth, *filiform*, *echinulate*, *beaded*, *spreading*, *arborescent*, *rhizoid*.
Lustre, *glistening*, *dull*.
Chromogenesis ______________ *photogenic*, *fluorescent*.
Odor, *absent*, *decided*, *resembling* ______________
Consistency, *butyrous*, *viscid*, *membranous*, *brittle*.
Medium, *grayed*, *browned*, *reddened*, *blued*, *greened*, *unchanged*.



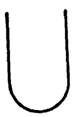
Medium:
Temperature
________ °C.
Age
________ d.

NUTRIENT BROTH: Temperature ______ °C. Age _____ d.
[illegible] *membranous*, *none*
[illegible] *persistent*, *none*,
Odor, *absent*, *decided*, *resembling* ______________
Sediment, *compact*, *flocculent*, *granular*, *flaky*, *viscid*.
Amount of sediment, *abundant*, *scanty*, *none*.



Medium:
Temperature
________ °C.
Age
________ d.

GELATIN STAB: Temperature ______ °C. Age _____ d.
Growth, *uniform*, *best at top*, *best at bottom*.
Line of puncture, *filiform*, *beaded*, *papillate*, *villous*, *arborescent*.
Liquefaction, *none*, *crateriform*, *infundibuliform*, *napiform*, [illegible] ____ d. complete in ____ d.
[illegible] days ______________
Method used ______________
Medium, *fluorescent*, *browned*, *unchanged*.



Medium:
Temperature
________ °C.
Age
________ d.

Fermentation Temperature ______ °C

Medium ____ containing ____ ____ and:	Monosaccharides							Disaccharides							Polysaccharides							Alcohols								Glucosides					
	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Melibiose	Cellobiose		Raffinose	Melezitose	Starch	Inulin	Dextrin	Glycogen		Glycerol	Erythritol	Arabitol	Adonitol	Mannitol	Sorbitol	Dulcitol		Salicin	Aesculin	Coniferin	a-Methyl Gluc.		
Gas in ____ fermentation tube																																			
Amt. CO_2 in Eldredge tube																																			
Reaction (pH) after ____ d.																																			
Titrable acidity in ml. of N/ ____ Na OH																																			

Studied by ____________________ Culture No. __________

Optimum conditions: Media ____________________ Temp. __________ °C.

Phases recorded on other charts: ____________________

Brief Characterization

As each of [illegible] marginal square by means of figure, as designated bel[illegible] not been determined, indicate with the letters U, V, and X according to the following code:

U, undetermined; V, variable; X, doubtful.

Morphological	Vegetative Cells	Form & arrangement: 1, streptococci; 2, diplococci; 3, micrococci; 4, sarcinae; 5, rods; 6, commas; 7, spirals; 8, branched rods; 9, filamentous	
		Diameter: 1, under 0.5μ; 2, between 0.5μ and 1μ; 3, over 1μ	
		Gram stain: 0, negative; 1, positive	
		Flagella. 0, absent; 1, peritrichic; 2, polar; 3, present but undetermined	
		Capsules: 0, absent; 1, present	
		Chains (4 or more cells): 0, absent; 1, present	
	Sporangia· 0, absent; 1, elliptical, 2, short rods: 3, spindled; 4, clavate, 5, drumsticks		
	Endospores: 0, absent, 1, central to excentric, 2, subterminal; 3, terminal		
Cultural	Agar Stroke	Growth: 0, absent; 1, abundant; 2, moderate; 3, scanty	
		Lustre· 1, glistening, 2, dull	
	Agar Colonies	Form: 1, punctiform; 2, circular (over 1 mm. diameter); 3, rhizoid; 4, filamentous; 5, curled; 6, irregular	
		Surface: 1, smooth, 2, contoured; 3, rugose	
	Gelatin Colonies	Form· 1, punctiform; 2, circular (over 1 mm.); 3, irregular, 4, filamentous	
		Surface: 1, smooth; 2, contoured; 3, rugose	
Physiological	Biologic relations[illegible] r animals but not for man, 3, for plants; 4, parasitic but not pathogenic; 5, [illegible]		
	Relation to free oxygen: 1, strict aerobe; 2, facultative anaerobe, 3, strict anaerobe, 4, microaerophile		
	In nitrate media: 0, neither nitrite nor gas; 1, both nitrite and gas; 2, nitrite but no gas; 3, gas but no nitrite		
	Chromogenesis 0, none; 1, pink, 2, violet; 3, blue; 4, green, 5, yellow; 6, orange; 7, red; 8, brown; 9, black		
	Other photic characters· 0, none; 1, photogenic; 2, fluorescent; 3, iridescent		
	Indole: 0, negative; 1, positive		
	Hydrogen sulfide: 0, negative; 1, positive		
	Hemolysis: 0, negative; 1, positive		
	Methemoglobin: 0, negative; 1, positive		
	Protein Liquefac-[illegible]	Gelatin: 0, negative; 1, positive	
		Casein: 0, negative; 1, positive	
		Egg albumin: 0, negative; 1, positive	
		Blood serum: 0, negative; 1, positive	
	Indicator Reduction	Litmus: 0, negative; 1, positive	
		Methylene blue: 0, negative; 1, positive	
		Janus green: 0, negative; 1, positive	
	Rennet production: 0, negative; 1, positive		

SUPPLEMEN

Temperature Relations

Medium ____________ pH ______
Optimum temperature for growth ________ °C.
Maximum temperature for growth ________ °C.
Minimum temperature for growth ________ °C.

THERMAL DEATH POINT: Time 10 minutes: ________ °C.
Medium ____________ pH ______

THERMAL DEATH TIME:
Medium ____________ pH ______

Temp.	Time	Temp.	Time
____ °C.	____ min.	____ °C.	____ min.
____ °C.	____ min.	____ °C.	____ min.
____ °C.	____ min.	____ °C.	____ min.
____ °C.	____ min.	____ °C.	____ min.
____ °C.	____ min.	____ °C.	____ min.

Chromogenesis

Gelatin ____________
Agar ____________
Potato ____________

Other Photic Characters

Photogenesis on ____________
Iridescence on ____________
Fluorescence in ____________

Relation to Reaction (pH) of Medium

Medium ____________
Optimum for growth: *about pH* ____________
Limits for growth: *from pH* ______ *to* ______

Relation to Free Oxygen

Method ____________
Medium ____________ Temp. ______ °C.
Aerobic growth *absent, present, better than anaerobic growth, microaerophilic*
Anaerobic growth: *absent, occurs in presence of glucose, of sucrose, of lactose, of nitrate; better than aerobic growth*
Additional data: ____________

Milk

Temperature ______ °C.

Reaction:	___ d. ___;	___ d. ___;	___ d. ___
Acid curd:	___ d. ___;	___ d. ___;	___ d. ___
Rennet curd:	___ d. ___;	___ d. ___;	___ d. ___
Peptonization:	___ d. ___;	___ d. ___;	___ d. ___

Litmus Milk

Temperature ______ °C

Reaction:	___ d. ___;	___ d. ___;	___ d. ___
Acid curd:	___ d. ___;	___ d. ___;	___ d. ___
Rennet curd:	___ d. ___;	___ d. ___;	___ d. ___
Peptonization:	___ d. ___;	___ d. ___;	___ d. ___

Reduction of litmus *begins in* ____ *days, ends in* ____ *days*

PATHOLOGY

Animal Inoculation

Medium used ____________ Age of culture ______ Amount ______ Incubation period ______

		Whole culture			Cells			Filtrate		
Animal										
Type of Injection	Subcutaneous	*								
	Intraperitoneal									
	Intravenous									
	Per os									

* In each instance where pathogenicity is observed, indicate location of lesion, and type, *e.g.* edema, histolysis, gas, hemorrhage, ulcer, diphtheritic, etc.

Antigenic Action

Animal ____________ Medium used ____________ Age of culture ______
Type injection ____________ Number of injections ______
Culture causes production of *cytolysins, agglutinins, precipitins, antitoxin.*
Specificity: Antibodies produced effective against other antigens as follows ____________

Immune sera from ____________
____________ effective against this organism as antigen

This DESCRIPTIVE CHART presented at the annual meeting of the SOCIETY OF AMERICAN
Prepared by a sub-committee consisting

TARY DATA

Action on Erythrocytes

Cells: ______
Method: *plate, broth, filtrate*
Hemolysis: *negative, positive*
Methemoglobin: *negative, positive*

Production of Indole

Medium ______
Test used ______
Indole *absent, present in* ______ *days*

Production of Hydrogen Sulfide

Medium ______
Test used ______
H_2S *absent, present in* ______ *days*

Action on Nitrates

Medium ______ Temp. ______ °C.
Nitrite: ___d.___; ___d.___; ___d.___; ___d.___
Gas (N_2): ___d.___; ___d.___; ___d.___; ___d.___
Medium ______ Temp. ______ °C.
Nitrite: ___d.___; ___d.___; ___d.___; ___d.___
Gas (N_2): ___d.___; ___d.___; ___d.___; ___d.___
Ammonia production (in amino-N-free nitrate medium): *negative, positive*
Complete disappearance of nitrate in ______ medium: *negative, positive*
D[illegible] 2 p.p.m. nitrite in ______ medium:
[illegible]

Reduction of Indicators

Medium ______ pH ______ Temp. ______ °C.

Indicator	Conc.	Reduction:	
______	______ %	___ hr. ___;	___ hr. ___
______	______ %	___ hr. ___;	___ hr. ___
______	______ %	___ hr. ___;	___ hr. ___
______	______ %	___ hr. ___;	___ hr. ___

Staining Reactions

Gram: ___ d. ___; ___ d. ___; ___ d. ___; ___ d. ___
Method ______
Spores: Method ______
Capsules: Method ______
Medium ______
Flagella: Method ______
Special Stains ______
______
______

Additional Tests

Methyl red: *negative, positive*
Voges-Proskauer: *negative, positive*
Growth in sodium citrate: *absent, present*
Growth in uric acid: *absent, present*
Hydrolysis of starch: *complete (iodine colorless); partial (iodine reddish-brown); none (iodine blue)*
Nitrogen obtained from the following compounds: ______
______
______
______

SPECIAL TESTS

BACTERIOLOGISTS, Dec. 28, 1934, by the Committee on Bacteriological Technic.
of M. W. Jennison and H. J. Conn.

Glossary of Terms Used on the Descriptive Chart

A number of scientific terms are used on the chart (page 124) to describe the various characteristics of organisms growing on different media. These terms, together with their definitions, are as follows:

Acid curd, coagulation of milk due to acid production.

Adherent, as applied to sporangium wall, indicating that remnants of sporangium remain attached to endospore for some time.

Aerobic, growing in the presence of free oxygen; strictly aerobic, growing only in the presence of free oxygen.

Agglutinin, an antibody having the power of clumping suspensions of bacteria.

Anaerobic, growing in the absence of free oxygen; strictly anaerobic, growing only in the absence of free oxygen; facultative anaerobic, growing both in presence and in absence of oxygen.

Antibody, a specific substance produced by an animal in response to the introduction of an antigen.

Antigen, a substance which, when introduced into an animal body, stimulates the animal to produce specific bodies that react or unite with the substance introduced.

Antigenic action, behavior as an antigen.

Antitoxin, an antibody having the power of uniting with or destroying a toxic substance.

Arborescent, branched, tree-like in growth.

Aseptically, without permitting bacterial contamination.

Autotrophic, able to grow in absence of organic matter.

Bacteriostasis, prevention of bacterial growth, but without killing the bacteria.

Beaded, in connection with stab or stroke culture, showing separate or semiconfluent colonies along the line of inoculation.

Bipolar, at both poles or ends of the bacterial cell.

Brittle, of dry growth; friable under the platinum inoculating needle.

Butyrous, having butter-like consistency.

Capsule, a gelatinous envelope surrounding the cell membrane of some kinds of bacteria.

Chains, four or more bacterial cells attached end to end.

Chromogenesis, the production of color.

Clavate, club-shaped.

Compact, referring to sediment in the form of single fairly tenacious mass.

Complement, a nonspecific enzyme-like substance, destroyed if subjected to heat (56°C. or over), which occurs in blood serum and is necessary, in conjunction with a specific antibody, in order to bring about cytolysis.

Concentrically ringed, marked with rings, one inside the other:

Contoured, having an irregular, smoothly undulating surface, like that of a relief map.

Crateriform, referring to a saucer-shaped liquefaction of the medium.

Cuneate, wedge-shaped.

Curled, composed of parallel chains in wavy strands, as in anthrax colonies.

Cytolysin, an antibody causing cytolysis.

Cytolysis, a dissolving action on cells.

Diastatic action, the conversion of starch into simpler carbohydrates, such as dextrins or sugars, by means of diastase.

Diphtheritic, diphtheria-like.
Dissociation, separation of characters, usually referring to phase variation (*q.v.*).

Echinulate, showing a growth along the line of inoculation with toothed or pointed margins.
Edema, accumulation of fluid in a part of an animal body.
Effuse, of thin growth; veily; unusually spreading.
Endospores, thick-walled spores formed within the bacteria; *i.e.*, typical bacterial spores like those of *B. anthracis* or *B. subtilis*.
Endotoxin, a toxic substance produced within a microorganism and not excreted.
Enzyme, a chemical ferment produced by living cells.
Erose, irregularly notched.
Excentric, slightly to one side of the center, between the positions denoted central and subterminal.
Exogenous, originating outside of the organism.
Exotoxin, a toxic substance excreted by a microorganism and hence found outside the cell body.

Facultative anaerobe, see anaerobic.
Filamentous, composed of long, irregularly placed or interwoven threads.
Filaments, as applied to morphology of bacteria, thread-like forms, generally unsegmented; if segmented, to be distinguished from chains (*q.v.*) by the absence of constrictions between the segments.
Filiform, in stroke or stab cultures, having a uniform growth along the line of inoculation.
Flagellum (pl.-la), a motile, whip-like attachment; an organ of locomotion.
Flaky, in reference to sediment, having the form of numerous separate flakes.
Flocculent, containing small adherent masses of bacteria of various shapes floating in the culture fluid.
Fluorescent, having one color by transmitted light and another by reflected light.

Gonidia, asexual spores.
Gonidial, referring specifically to a bacterial phase producing gonidia-like bodies.
Granular, composed of small granules.

Hemolysin, an antibody causing hemolysis.
Hemolysis, a dissolving action on red blood corpuscles.
Hemorrhage, an escape of blood from the vessels.
Histolysis, a breaking down of tissues.
Hydrolysis of starch, destruction of starch by the formation of a chemical union with water; includes diastatic action but is the more general term.

Immune serum, an animal fluid containing an antibody.
Inactivate, to destroy the complement by heat.
Infundibuliform, in form of a funnel or inverted cone.
Intraperitoneal, within the peritoneum.
Intravenous, within a vein.
Iridescent, exhibiting changing rainbow colors in reflected light.

Lesion, a local injury or morbid structural change.
Lobate, having lobes or rounded projections.

Maximum temperature, temperature above which growth does not take place.
Membranous, of thin growth; coherent, like a membrane.
Metabolite, a substance produced by metabolism.
Microaerophilic, growing best in presence of small quantities of oxygen.
Minimum temperature, temperature below which growth does not take place.
Mucoid, mucus-like, referring specifically to a bacterial phase producing slimy growth.
Mycelioid, referring to colonies having the radiately filamentous appearance of mold colonies.

Napiform, having the form of a turnip.

Ontogenetic, pertaining to the life history of an individual.
Opalescent, resembling the color of an opal.
Opaque, not allowing light to pass through.
Optimum temperature, temperature at which growth is most rapid.

Papillate, beset with small nipple-like processes.
Parasitic, deriving its nourishment from some living animal or plant upon which it lives and which acts as host; not necessarily pathogenic.
Pathogenic, not only parasitic (*q.v.*) but also causing disease to the host.
Pellicle, bacterial growth forming either a continous or an interrupted sheet over the culture fluid.
Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.
Peritrichiate, applied to the arrangement of flagella, indicates that they are distributed over the entire surface of an organism.
Peritrichic, having flagella in peritrichiate arrangement.
Per os, through the mouth.
Persistent, lasting many weeks or months.
Phase variation, the separation of a species into strains, having different characters.
Photogenic, glowing in the dark; phosphorescent.
Polar, at the end or pole of the bacterial cell.
Precipitin, an antibody having the power of precipitating soluble proteins.
Pulvinate, cushion-shaped.
Punctiform, very small, but visible to naked eye; under 1 mm. in diameter.

Raised, of thick growth with abrupt or terraced edges.
Reduction, removal of oxygen or its equivalent from a chemical compound, or the addition of hydrogen or its equivalent. Refers to the conversion of nitrate to nitrite, ammonia, or free nitrogen; also to the decolorization of litmus.
Rennet curd, coagulation of milk due to rennet or rennet-like enzymes, distinguished from acid curd by the absence of acid.
Rhizoid, growth of an irregular branched or root-like character, as *B. mycoides.*
Ring, growth at the upper margin of a liquid culture, adhering to the glass.
Rugose, wrinkled.

Saccate, liquefying in form of an elongated sac, tubular, cylindrical.
Saprophitic, unable to grow in the absence of organic matter, *i.e.*, not autotrophic (*q.v.*); but not parasitic, as a living host is unnecessary.
Sensitize, to render sensitive to a protein.
Sepsis, a state of infection.

Spindled, larger at the middle than at the ends. Applied to sporangia, it refers to the forms frequently called clostridia.

Sporangia, cells containing endospores.

Spreading, growing much beyond the line of inoculation, *i.e.*, several millimeters or more.

Stratiform, liquefying to the walls of the tube at the top and then proceeding downward horizontally.

Strict aerobe, see aerobic.

Strict anaerobe, see anaerobic.

Subcutaneous, under the skin.

Subterminal, situated toward the end of the cell but not at the extreme end, *i.e.*, between the positions denoted excentric (*q.v.*) and terminal.

Synergism, cooperative action.

Thermophilic, growing best at high temperatures, *i.e.*, 50°C. or over.

Toxic, poisonous.

Transient, lasting a few days.

Translucent, allowing light to pass through without allowing complete visibility of objects seen through the substance in question.

Trituration, thorough grinding in a mortar.

Truncate, ending abruptly; square.

Turbid, cloudy with flocculent particles, *i.e.*, cloudy plus flocculent.

Ulcer, an open sore caused by infection.

Villous, having short, thick, hair-like processes on the surface, intermediate in meaning between papillate and filamentous (*q.v.*).

Virulence, degree of pathogenicity (referring to infectiousness).

Virus, a living cause of disease, often referring to one too small to be seen with the microscope.

Viscid, referring to growth that follows the needle when touched and withdrawn; or to sediment that on shaking rises as a coherent swirl.

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CHAPTER VIII

EFFECT OF ENVIRONMENT UPON BACTERIA

It is well known that the life activities of organisms are conditioned by their environment. Any marked change in the environment produces a corresponding change in the morphological and physiological characteristics of the organisms. Bacteria quickly adapt themselves to the changed conditions, and for this reason, are able to withstand great variations in the environment. In this respect they differ markedly from higher plant and animal cells. By understanding the various physical and chemical factors controlling survival and multiplication, bacterial activity may be either increased, decreased, or destroyed entirely.

Bacteria multiply normally by binary or transverse fission. The rate at which division takes place can be made to vary widely. Any alteration in the time between consecutive cell divisions (generation time) indicates that one or more environmental factors have changed.

Destruction of bacteria by such agents as heat, light, osmotic pressure, desiccation, reaction of medium, toxic action of certain ions, or influence of heavy metals appears to follow a monomolecular reaction, where only one substance undergoes change and in which the velocity of the reverse reaction is negligible. The disinfection process does not take place suddenly but is a gradual operation in which the number of organisms killed in unit time is greater at the beginning and becomes less and less as the action proceeds. If the numbers of survivors in unit time are plotted against time and curves drawn, it will be found that the points lie upon smooth curves. On the other hand, if the logarithms of the number of surviving organisms are plotted against time, the points fall on a straight line. This is a general rule applicable to all agents employed for the destruction of microorganisms. As is true with all rules, there are a few exceptions. Some investigators believe that the processes employed for the destruction of bacteria are so complicated that they cannot be interpreted mathematically.

EFFECT OF TEMPERATURE

Bacteria are able to survive wide limits of temperature, but the range at which they can grow and carry on their life ~~activities falls~~ between 0 and 90°C. There exists for every organism a maximum, a minimum, and an optimum temperature for growth.

Maximum Growth Temperature.—The maximum temperature may be defined as the highest temperature at which growth and multiplication will occur, when the other environmental factors are kept constant. The psychrophilic organisms, *i.e.*, those that grow at low temperatures, do not develop well even at room temperature. Saprophytic mesophilic organisms show a maximum at about 30°C. The majority of the parasitic forms fall between 40 and 50°C. Some of the thermophilic (heat-loving) bacteria may show growth at temperatures as high as 60 to 70°C., or even higher.

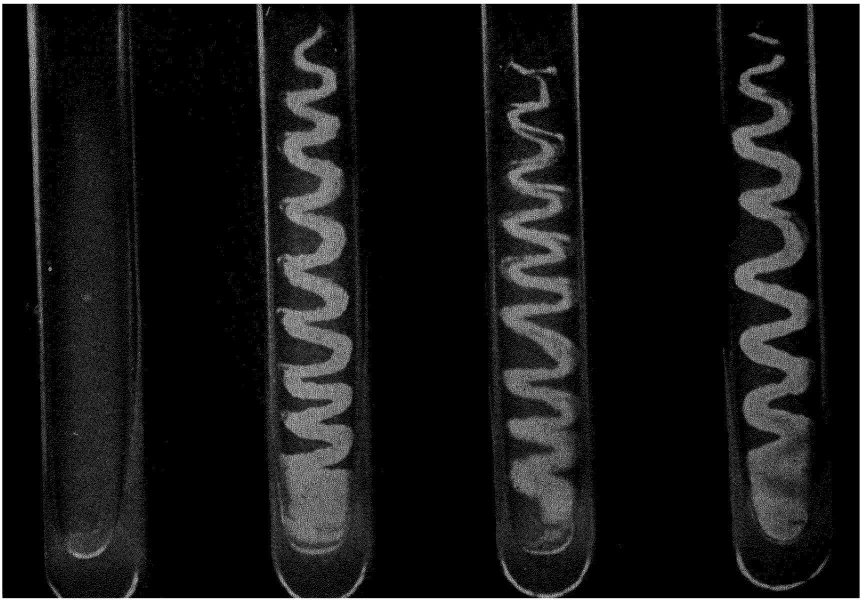


FIG. 96.—Effect of incubation at 15°C. upon growth. *A*, *Bacillus viridulus;* *B*, *Bacillus subtilis;* *C*, *Escherichia coli;* *D*, *Flavobacterium brunneum.*

Optimum Growth Temperature.—The optimum temperature is the most favorable temperature for growth. The psychrophilic or cold-loving organisms have an optimum temperature below 18 to 20°C. These organisms are found in cold lake and spring waters and in brines kept under cold-storage conditions. This group includes many of the pigment-producing bacteria.

The mesophilic organisms have an optimum temperature of 18 to 45°C. Most of them have an optimum growth temperature of 18 to 25°C. The saprophytic mesophiles grow best at temperatures of 18 to 25°C.; the parasitic mesophiles grow best at the temperature of the host.

The thermophilic bacteria vary greatly in their temperature optima. Many possess an optimum temperature of about 55°C. They occur in soil, in manure, in excreta, in decaying organic matter, etc. Owing to their great resistance to heat, they are the source of considerable trouble in the canning industry.

The effect of different temperatures of incubation on the growth of *Bacillus viridulus*, *B. subtilis*, *Escherichia coli*, and *Flavobacterium brunneum* is shown in Figs. 96, 97, 98, and 99.

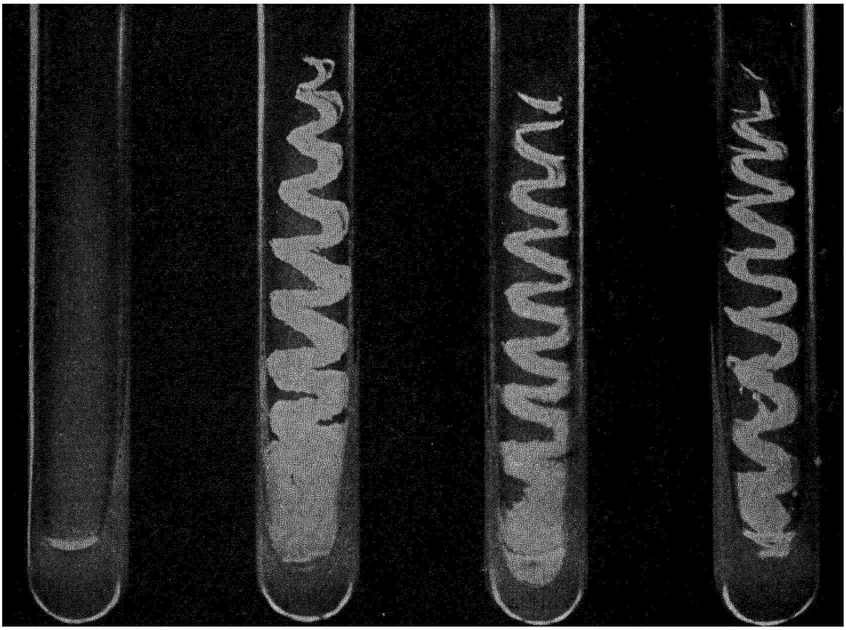


FIG. 97.—Effect of incubation at 25°C. upon growth. *A*, *Bacillus viridulus*; *B*, *Bacillus subtilis*; *C*, *Escherichia coli*; *D*, *Flavobacterium brunneum*.

Minimum Growth Temperature.—The minimum temperature is the lowest temperature where growth and multiplication will occur under certain specified conditions. This temperature will also show variation when one or more environmental factors are changed.

The multiplication rate of an organism is exceedingly slow at the minimum temperature. As the temperature is lowered from the optimum to the minimum, the rate of multiplication becomes progressively less and less. Beyond the minimum temperature multiplication ceases entirely.

Growth-temperature Range.—This is defined as the number of degrees, or magnitude of the range, between the minimum and maximum growth temperatures. With some organisms this range is very narrow; with others it is very wide.

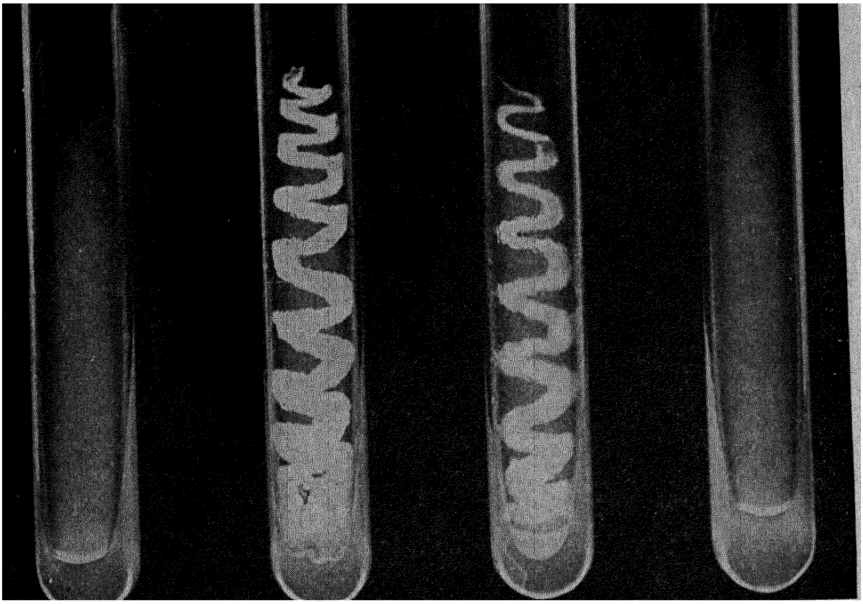


FIG. 98.—Effect of incubation at 37°C. upon growth. *A, Bacillus viridulus; B, Bacillus subtilis; C, Escherichia coli; D, Flavobacterium brunneum.*

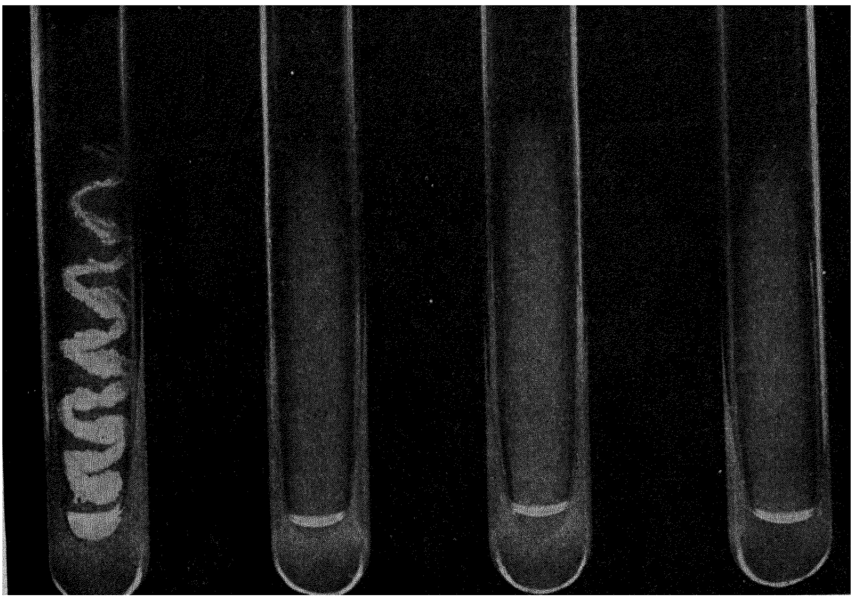


FIG. 99.—Effect of incubation at 55°C. upon growth. *A, Bacillus viridulus; B, Bacillus subtilis; C, Escherichia coli; D, Flavobacterium brunneum.*

The temperature relations of the three classes of organisms are as follows:

1. Psychrophilic:
   Minimum = 0°C. Optimum = 15 to 20°C. Maximum = 30°C.
2. Mesophilic:
   Minimum = 5 to 25°C. Optimum = 37°C. Maximum = 43°C.
3. Thermophilic:
   Minimum = 25 to 45°C. Optimum = 50 to 55°C. Maximum = 60 to 90°C.

THERMAL DEATH POINT

The thermal death point may be defined as that temperature at which an organism is killed after an exposure period of 10 min., under certain specified conditions. The term is not strictly correct because organisms subjected to unfavorable conditions are not all killed in the same length of time. As is true in all populations some members are more resistant than others. Therefore, it is more correct to use the terms thermal death rate or thermal death time.

The various factors that should be specified in reporting a thermal death rate include (1) the percentage of moisture present in the medium, (2) the hydrogen-ion concentration of the medium, (3) the composition of the medium, (4) the age of the cells, and (5) the presence or absence of spores in a culture of a spore-forming organism. If one or more of the factors are changed the thermal death rate will also change. Therefore, all the foregoing factors must be given in order that any importance may be attached to the results.

Water Content of the Medium.—Within limits, the greater the percentage of water in the medium the lower will be the thermal death rate. Moist heat is more effective as a sterilizing agent than dry heat. Dry egg albumin may be heated to a point where it decomposes without showing any appreciable coagulation. As the percentage of moisture is increased, the temperature of coagulation becomes progressively less. This may be seen in Table 6.

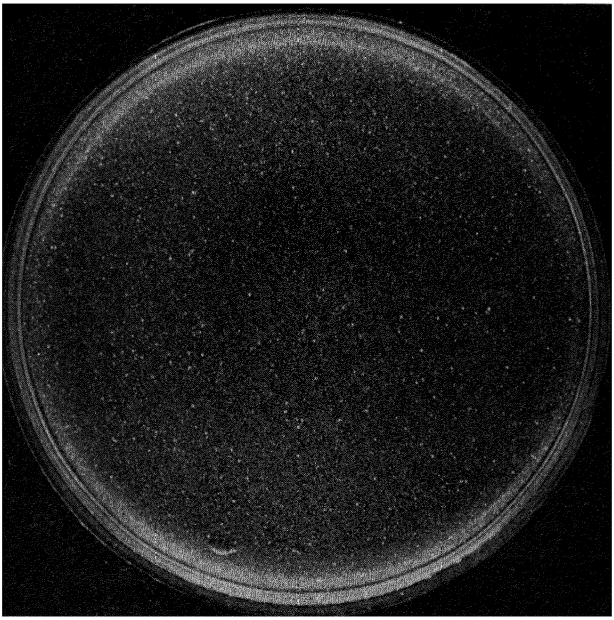
TABLE 6.—RELATION BETWEEN MOISTURE CONTENT AND TEMPERATURE OF COAGULATION OF EGG ALBUMIN

Amount of Water, Per Cent	Temperature of Coagulation, °C.
50	56
25	74– 80
18	80– 90
6	145
0	160–170

pH of the Medium.—Most organisms are more easily destroyed in acid or alkaline solutions than in a neutral environment. In general,

the greater the degree of acidity or alkalinity the lower will be the thermal death rate. A neutral (pH7.0) solution should be used.

Composition of the Medium.—The composition of the substrate plays a very important role in the results obtained for the thermal death rate. Media containing high concentrations of proteins or albuminous substances usually show higher thermal death rates. This is owing to the fact that the proteins form a film around the organisms protecting them from unfavorable influences.

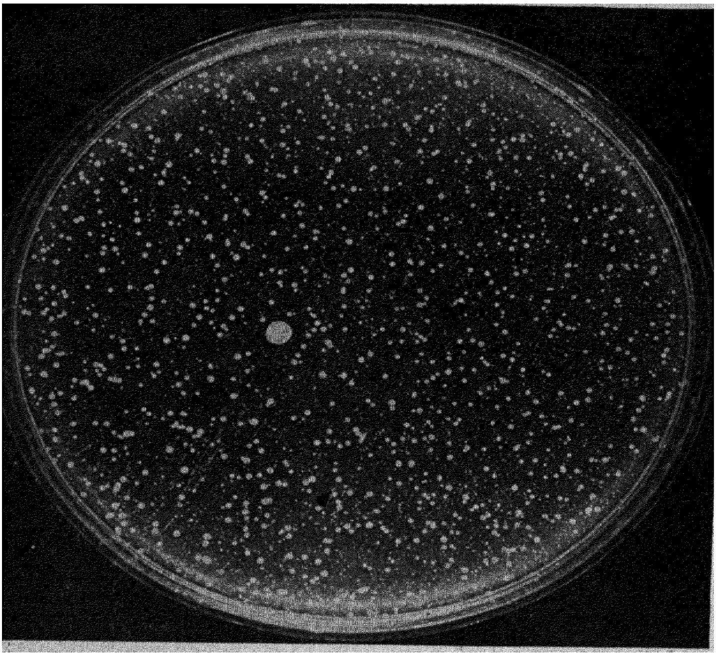


A

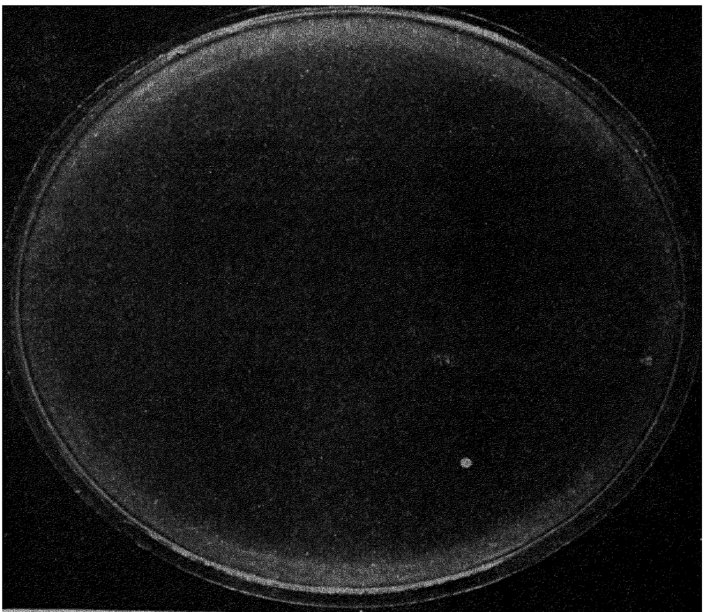
Fig. 100.—Thermal death rate of *Staphylococcus aureus*. *A*, 65°C.; *B*, 70°C.; *C*, 75°C.

Age of the Cells.—The age of the cells also influences the thermal death rate. Very old cells that have become devitalized are said to be more susceptible to heat than younger cells. On the other hand very young bacteria, *i.e.*, cells that have not fully matured, might be less resistant to heat than older cells. It is best to use 24-hr. old cultures for the test.

Presence of Spores.—Nonspore-forming bacteria and the vegetative forms of the spore bearers are usually killed by moist heat at temperatures of 60 to 70°C. Spores can withstand temperatures of 100°C., and higher. Since spores are produced under unfavorable environmental conditions, they will be more numerous in old cultures. In reporting the thermal death rate of a spore-forming organism precautions should be taken to make sure that spores are present in the culture.



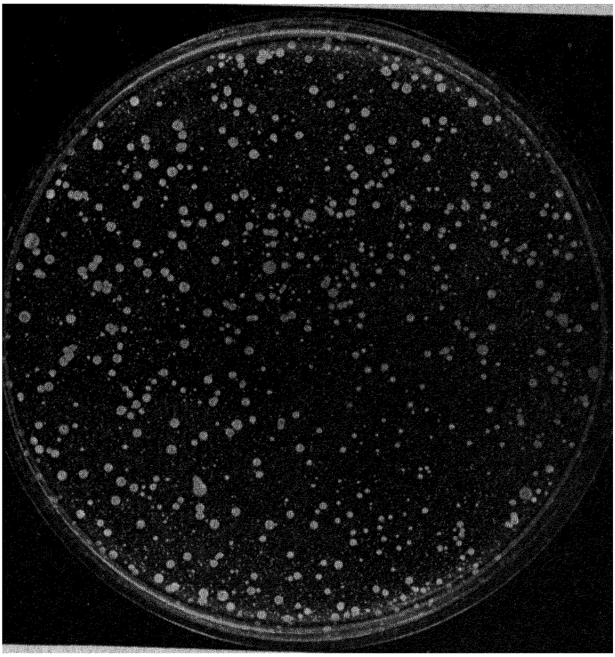
B



C

FIG. 100.—For descriptive legend see p. 137.

Lamanna (1942) found that the heat resistance of both vegetative cells and spores was related to the maximum temperature of growth. Members of the genus *Bacillus* (*B. subtilis*, *B. agri*, *B. vulgatus*, *B. mesentericus*, *B. cereus*, *B. mycoides*, and *B. megatherium*) were separated into three groups on the basis of heat tolerance by spores. The thermophiles produced spores of greatest heat resistance and showed the highest growth temperature. The species with a maximum below 50°C. possessed spores of least resistance, whereas nonthermophilic types with maxima between 50 to 60°C. had spores of intermediate heat resistance.



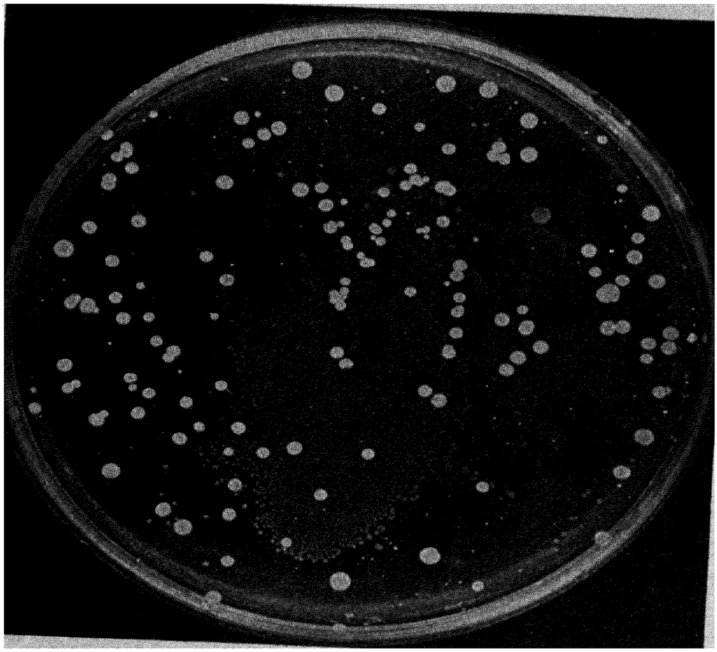
A

FIG. 101.—Thermal death rate of *Bacillus megatherium*. *A*, 90°C., *B*, 95°C.; *C*, 100°C.

Results obtained from the determinations of thermal death rates are very valuable in applied bacteriology and especially in the canning industry. Such results aid the canner in determining the temperatures required to process certain canned foods.

It is the usual practice to isolate the organism or organisms causing the spoilage of a certain kind of food and determine their thermal death rates under similar environmental conditions. The results may then be used as a guide in determining the temperature required to process the food.

The thermal death rates for *Staphylococcus aureus* and *Bacillus megatherium* are shown in Figs. 100*A*, *B*, *C*, and 101*A*, *B*, *C*.



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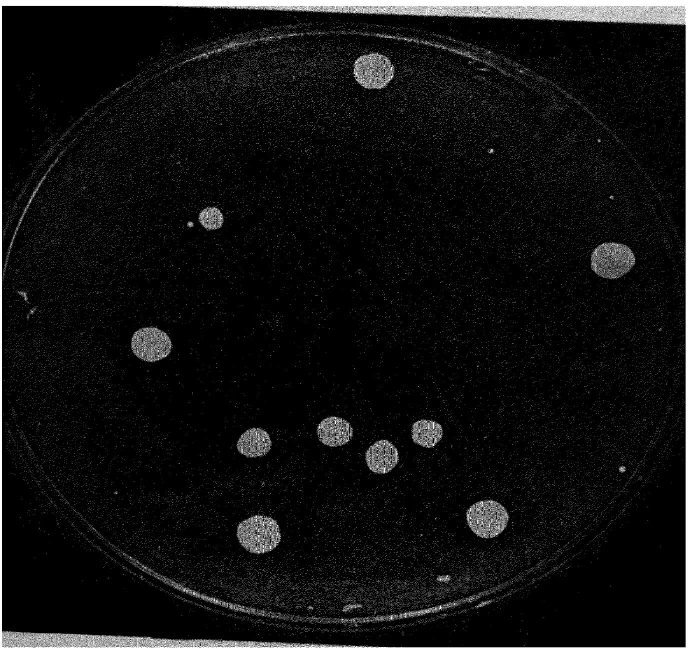


C

FIG. 101.—For descriptive legend see p. 139.

EFFECT OF ULTRAVIOLET LIGHT

In general, the light rays in the ultraviolet region produce a toxic action on bacteria. The growth of an organism may be retarded or completely destroyed, depending upon the length of the rays and the period of exposure.

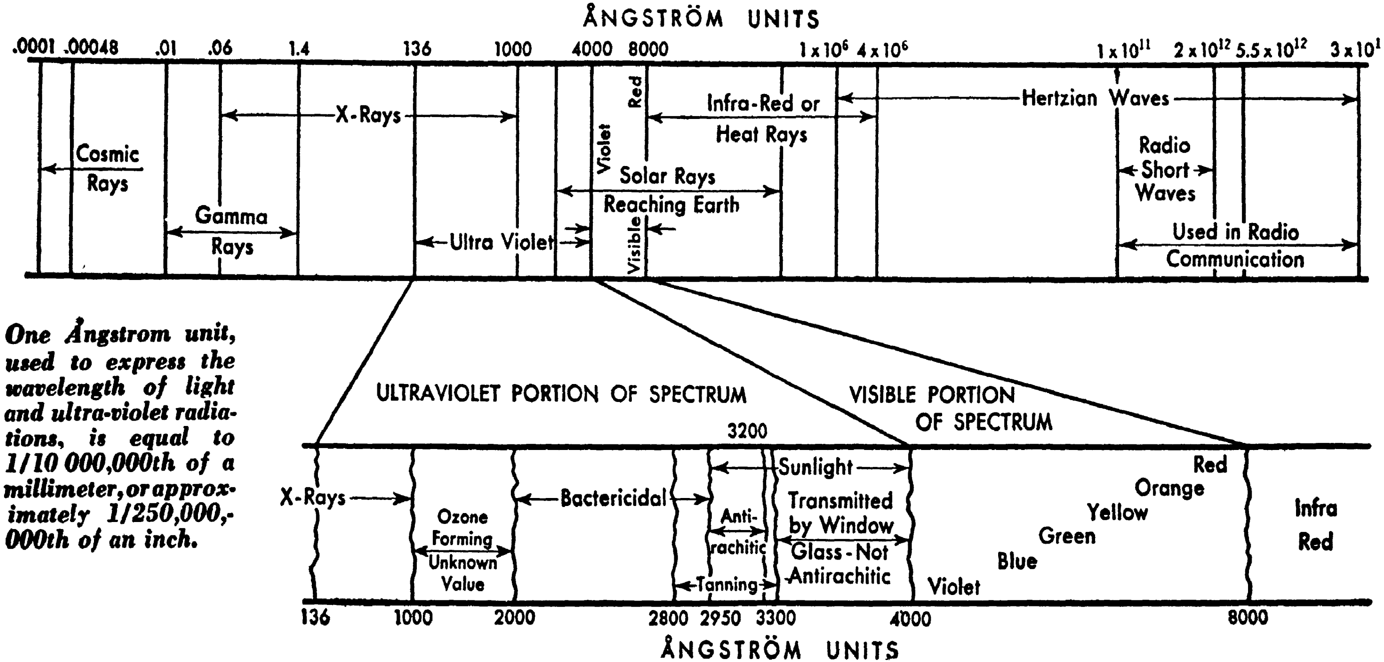
With the possible exception of a few species, bacteria do not produce chlorophyll. A few sulfur bacteria elaborate a pigment known as bacteriopurpurin, which appears to function in bacteria in a manner similar to chlorophyll in higher plants. These organisms are not harmed but benefited by light rays. However, the great majority of the bacterial species known do not exhibit any photosynthetic action. These organisms are harmed by exposure to ultraviolet light.

Sharp (1939) seeded agar plates from cultures of several species of nonspore-bearing organisms and one culture containing spores and vegetative cells of *B. anthracis*. Immediately after streaking, the plates were irradiated with light rays of 2537 Å. until the organisms were reduced to a 10 per cent survival (90 per cent killed). The results showed that spores of *B. anthracis* required approximately twice the exposure as vegetative cells to produce the same percentage of reduction. Hercik (1937) also found that twice as much incident energy was required to destroy spores of *B. megatherium* as the vegetative cells.

Smithburn and Lavin (1939) irradiated the organism of tuberculosis (*Mycobacterium tuberculosis*) with sublethal doses of monochromatic light of 2537 Å. and found that the organisms gradually lost their virulence and finally became avirulent without being killed. These avirulent organisms were still capable of inducing a demonstrable immunity whereas organisms killed by the same light rays did not induce a measurable immunity. Organisms killed by light rays still possessed acid-fast properties.

Spores of molds have also been treated with ultraviolet light and found to be susceptible to the same rays that are toxic to bacteria. Hollaender and Emmons (1939) reported that spores of *Trichophyton mentagrophytes* isolated from "athlete's foot" were destroyed by light rays of 2537 to 2650 Å.

Viruses and bacteriophages are also sensitive to light rays. Jungeblut (1937) and Toomey (1937) found that the virus of poliomyelitis (infantile paralysis) was destroyed by light rays in the ultraviolet region. Similar results were reported by Levaditi and Voet (1935) for herpes virus and *E. coli* bacteriophage. Wells and Brown (1936) sprayed influenza virus into a testing chamber, followed by exposure to ultraviolet radiation. They reported that the virus was completely inactivated. Kendall and Colwell (1940) showed that bacteriophages specific for several



One Ångstrom unit, used to express the wavelength of light and ultra-violet radiations, is equal to 1/10 000,000th of a millimeter, or approximately 1/250,000,-000th of an inch.

FIG. 102.—Spectrum charts. (*From The Westinghouse Sterilamp and the Rentschler-James Process of Sterilization, courtesy of the Westinghouse Electric & Manufacturing Company, Inc.*)

strains of *E. coli*, *Shigella paradysenteriae*, and *Staphylococcus aureus* were destroyed within 1 min. by exposure to ultraviolet light emitted by a quartz mercury vapor lamp exposed at a distance of 1 cm. from the face of the lamp. Ingredients of broth associated intimately with bacteriophage particles in the same medium, or interposed as a screen between pure phage and light source, prevented their destruction.

Ultraviolet rays are used commercially for the destruction of bacteria, yeasts, and molds in various foodstuffs such as sugar, meats, and bakery

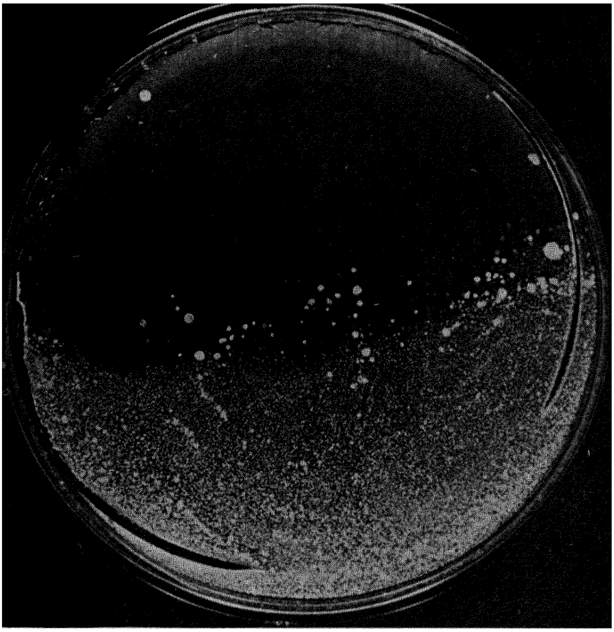


FIG. 103.—Effect of ultraviolet rays on the growth of *Bacillus subtilis*. One-half of plate protected from light.

products. Hall and Keane (1939) reported that spores of *Bacillus stearothermophilus*, which are commonly found in sugar and which are the cause of serious trouble in many food preparations containing sugar, may be destroyed by irradiation of the sugar crystals.

Many hospital rooms and operating rooms are irradiated to sterilize or greatly decrease the numbers of organisms in the air. Air sanitation is closely analogous to water sanitation (Robinson, 1939). Its purpose is substantially the same, namely, to make the air in confined spaces more safe under the particular circumstances of its use, and to guard against the possibility that air-borne organisms may cause clinical infections. In hospital operating rooms the primary objective is to reduce the risk from pathogenic organisms settling from the air on the surface of open

incisions, on instruments, or on the hands of the operating personnel from which they might be transferred into open wounds. Robertson, Doyle, Tisdall, Koller, and Ward (1939) sprayed bacteria into an experimental room and showed that their spread from cubicle to cubicle was prevented by the application of ultraviolet rays of 2537 Å.

Irradiation of Culture Media.—The observations already reported are limited to the action of the light rays on microorganisms and their spores. It has been shown that culture media, when exposed to ultraviolet light, become less suited for bacterial growth. Bedford (1927), after a series of experiments, concluded that the irradiation of culture media caused the formation of hydrogen peroxide. The presence of this compound in media produces a toxic action on bacteria. The concentration of peroxide accumulating in media depends upon the wave length of the light rays and the period of exposure.

Pratt (1936), Baumgartner (1936), and others have shown that the irradiation of carbohydrate solutions and carbohydrate media caused a shift in the pH towards more acid conditions. The sequence of changes appeared to be polysaccharides → disaccharides → monosaccharides → alcohols, aldehydes, ketones, and acids. A considerable portion of the acid formed was formic acid. Baumgartner stated that neutralization of the acid restored the ability of the culture media to support growth of the bacteria.

Only those light rays which are absorbed can bring about any chemical change. Most proteins in solution show marked absorption bands in the ultraviolet region. The germicidal rays measure from 2000 to 2950 Å. in length with a maximum effect at about 2537 Å.

EFFECT OF OSMOTIC PRESSURE

Osmosis may be defined as a kind of diffusion that takes place between two miscible fluids separated by a permeable membrane where the conditions on the two sides of the membrane tend to become equal.

The term osmotic pressure refers to the unbalanced pressure that gives rise to the phenomena of diffusion and osmosis, as in a solution in which there are differences of concentration.

Plasmolysis.—The rate at which water passes into and out of cells is in part determined by the ratio that exists between the concentrations of electrolytes inside and outside of the cell membranes. Most cell contents exert a definite pressure on the cell membranes. If an organism is immersed in a solution having a higher osmotic pressure, water will leave the cell. This will continue until an equilibrium is established between the osmotic pressures inside and outside of the cell. If the initial difference in osmotic pressure between the inside and outside of the cell is sufficiently great, the cytoplasmic membrane will be drawn

in with the cytoplasmic contents and collect in the center of the cell. The cell is then said to be plasmolyzed and the process is called plasmolysis. The solution on the outside is hypertonic with respect to the solution on the inside of the cell.

Plasmoptysis.—If the concentration of the electrolytes in the solution on the outside of the cell is less than the concentration on the inside, water will be drawn through the membranes into the cell. The result in this case will be a swelling of the cell. If the initial difference in osmotic pressure between the solutions on the inside and outside of the cell is sufficiently great, the cell membranes will burst, releasing their contents. The cell is then said to be plasmoptyzed and the process is called plasmoptysis. In this case the solution on the outside is hypotonic with respect to the solution on the inside of the cell.

Isotonic Solutions.—If the concentration of electrolytes on the inside and that on the outside of the cell membranes are equal, there will be no difference in their osmotic pressures. The result will be neither shrinking nor swelling of the cell contents. The two solutions are then said to be isotonic because they have approximately the same ionic concentrations.

A great increase in the osmotic pressure of the surrounding solution is necessary before any toxic action is noted on bacteria. In this respect they differ markedly from higher plant and animal cells, which are very sensitive to relatively slight changes in the ionic concentrations of the outside medium.

The use of high osmotic pressures finds a practical application in the preservation of some foods from bacterial attack. This principle is employed in the preservation of jams, jellies, and condensed milk by means of sugar, and salted meats, corned beef, fish, etc., by the use of salt.

Marine Bacteria.—Marine bacteria differ from fresh-water organisms in that they are able to tolerate greater concentrations of salt. ZoBell and Feltham (1933) found that less than 10 per cent of the bacteria isolated from sea water are able to multiply in nutrient fresh-water media and a smaller number of bacterial species isolated from fresh water can multiply in media prepared with undiluted sea water.

ZoBell and Michener (1938) acclimatized marine bacteria to hypotonic solutions by gradually diluting the sea-water medium with each successive transfer of the cultures. Of 12 isolated species most of them could be acclimatized to 25 to 30 per cent sea-water media; below this concentration considerable difficulty and delay were encountered in making the bacteria grow.

All except three of the original cultures, which were kept in the refrigerator on undiluted sea-water agar, multiplied when transferred to fresh-water medium. The other three species multiplied when

transferred to 10 per cent sea-water medium. The old stock cultures adapted themselves better to hypotonic solutions than did cultures of the same organisms gradually acclimatized to decreasing concentrations of sea-water media.

This observation appears to be contrary to accepted beliefs with respect to the adaptability of organisms to changes in the environmental conditions. On second thought, however, other factors must be taken into consideration to make a correct interpretation of the facts. It is well established that young bacteria are more susceptible to adverse

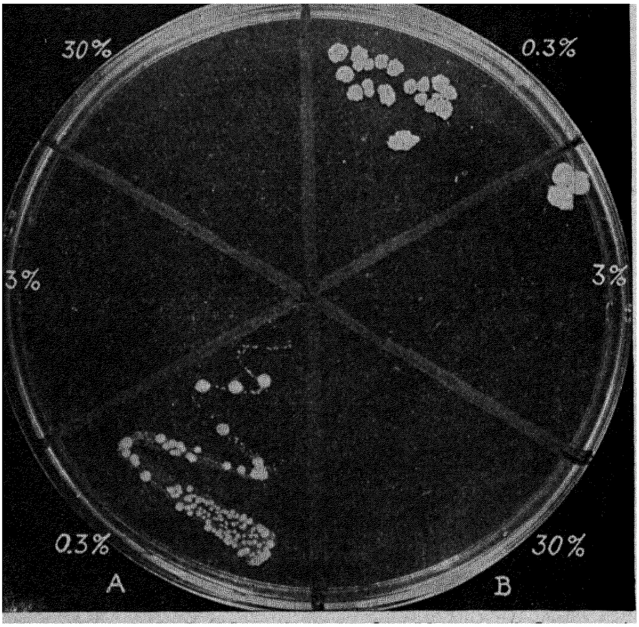


FIG. 104.—Osmosis. *A*, *Escherichia coli* exposed to 30 per cent, 3 per cent, and 0.3 per cent NaCl for 24 hr., then streaked onto the three sectors; *B*, *Bacillus subtilis* exposed to 0.3 per cent, 3 per cent, and 30 per cent NaCl for 24 hr., then streaked onto the three sectors.

environmental conditions than old ones. This has been shown by Sherman and Albus (1923) and others. Cultures gradually acclimatized to decreasing concentrations of sea water tend to keep the organisms in a physiologically young condition. On the other hand the parent stock cultures become physiologically old and senescent and less susceptible to changes in the environment. These cells are better able to adapt themselves to adverse conditions.

PRESERVATION OF BACTERIA

Organisms in the dry state are not capable of multiplication. Moisture is absolutely necessary for growth and multiplication. When

organisms are dried, they gradually die, the rate of death being dependent upon several factors. The cause of death is not definitely known. It may be due to a denaturation of the proteins of the protoplasm, to the destruction of the essential enzymes, or to other causes.

Spores are more resistant to desiccation than the vegetative cells producing them. They are able to withstand unfavorable environmental conditions for long periods of time.

Capsulated organisms are more resistant to drying than the non-capsule-producing bacteria. The mucilaginous deposit surrounding the organisms acts as a protective layer, decreasing the rate of desiccation.

Desiccated bacteria are more resistant to destructive agencies than the same organisms in the moist state. Much higher temperatures are required to coagulate the protoplasm of partly dried organisms than the same cells under normal conditions. Heat probably does not coagulate the protoplasm of completely dried bacteria. This explains why higher temperatures are required to sterilize glassware by the dry-air sterilizer than by the autoclave. Dried bacterial spores are also more resistant to adverse conditions than spores kept in the moist condition.

Bacteria for class use are generally preserved on nutrient agar slants. There are some exceptions, such as certain pathogenic organisms, that require the addition of serum, blood, and other special ingredients to the medium; anaerobic organisms that require the presence of tissue to remove the dissolved oxygen; certain soil organisms that require special inorganic media; etc. The toxic metabolic waste products secreted by organisms diffuse into the agar and away from the bacteria. Because of this property of agar, bacteria are able to survive longer than when grown in liquid media where they are constantly bathed by the toxic substances dissolved in the surrounding nutrient solution. Such cultures are referred to as stock cultures, because they are kept on hand by storing in a cool room or cupboard and used as needed for the preparation of transplants.

The maintenance of a large number of stock cultures of bacteria for use as needed requires frequent attention to prevent loss of the organisms. The method commonly employed for this purpose is to prepare transfers to suitable media at definite intervals before the media become too dehydrated and the bacteria are destroyed by the accumulation of waste products of metabolism. This requires not only a considerable amount of time but involves also the possible loss of certain biological, immunological, and cultural characteristics of the organisms.

Various methods are employed to preserve stock cultures and to maintain them in as near as possible their original state. According to Morton and Pulaski (1938) and Morton (1938) all the methods may be placed into either of two groups. In one group, the bacteria are preserved

by the prevention of slow drying; in the other, the organisms are preserved by rapid desiccation.

Preservation by Prevention of Slow Drying.—All the methods in this group attempt to preserve cultures by the application of some type of seal to the tubes to prevent or decrease drying.

The most important methods in this group are the following:

1. Cultures may be preserved by impregnating the cotton stoppers with paraffin and then inserting them into the tubes. The stoppers are easily removed by gently heating them in a flame to melt the paraffin. Sometimes the tops of the stoppers and test tubes are covered with melted sealing wax. This is not so satisfactory as paraffin because sealing wax is brittle and must be replaced each time the culture tubes are opened.

There are disadvantages to the use of paraffin and sealing wax. (*a*) The sealing materials are difficult to remove during the cleaning process. (*b*) The cultures are not protected from mold contaminations. Mold spores are frequently present on cotton stoppers. When the stoppers are sealed they soon become moistened by the evaporation of water from the medium. The water concentration soon becomes sufficiently great to permit germination of the spores. This results in the growth of hyphae, which penetrate through the cotton stoppers into the medium. (*c*) Many organisms are capable of dissociating when kept in sealed tubes. The organisms may undergo changes in colonial forms, in virulence, in their immunological specificities, and in other ways.

2. The tops of the tubes may be covered with paraffined paper, with tin or aluminum foil, or with rubber caps.

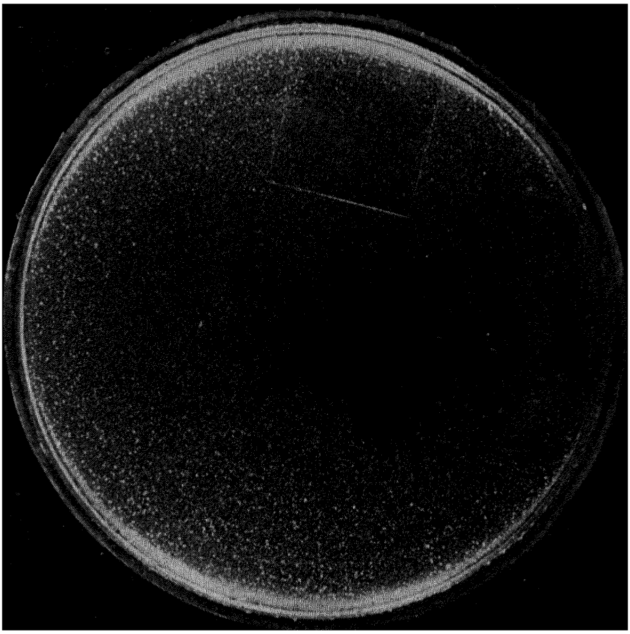
The objections given under the first method also apply here, especially contaminations due to molds.

3. The culture tubes may be sealed off as ampules by heating the open end in a blast lamp and drawing out the melted tops with a pair of forceps.

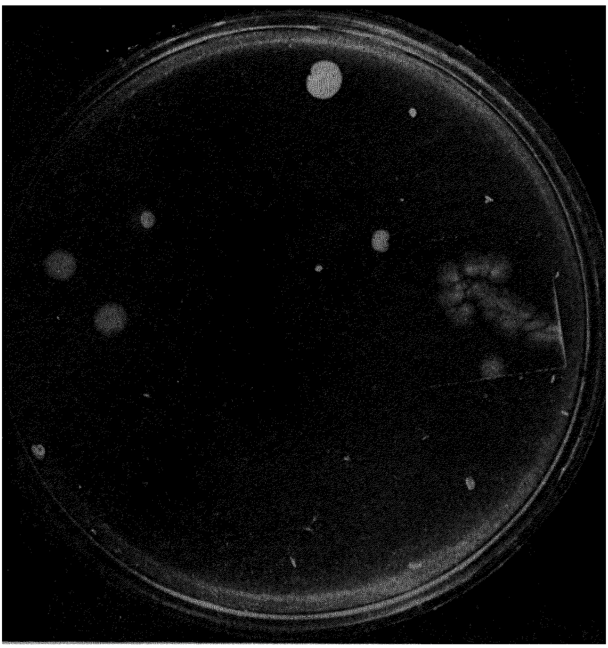
The disadvantages here are (*a*) the destruction of test tubes, (*b*) the difficulties encountered in opening the ampules, and (*c*) the cultures preserved in this manner also readily dissociate.

4. The culture media may be overlayered with sterile paraffin or mineral oil.

The cultures may be in broth or on agar slants. The broth cultures should be overlayered with mineral oil to a height of 1 cm. Agar slant cultures are first incubated until good growth appears and then covered with sterile mineral oil to a height of 1 cm. above the top of the slanted surface. Transplants are easily made by fishing off a loopful of the growth, touching the wire loop to the inner wall of the tube to drain off the excess oil, and then streaking over the surface of fresh medium.



A



B

FIG. 105.—Effect of desiccation on *Escherichia coli*. *A*, control; *B*, dried 48 hr.; *C*, dried 96 hr.; *D*, dried 144 hr.

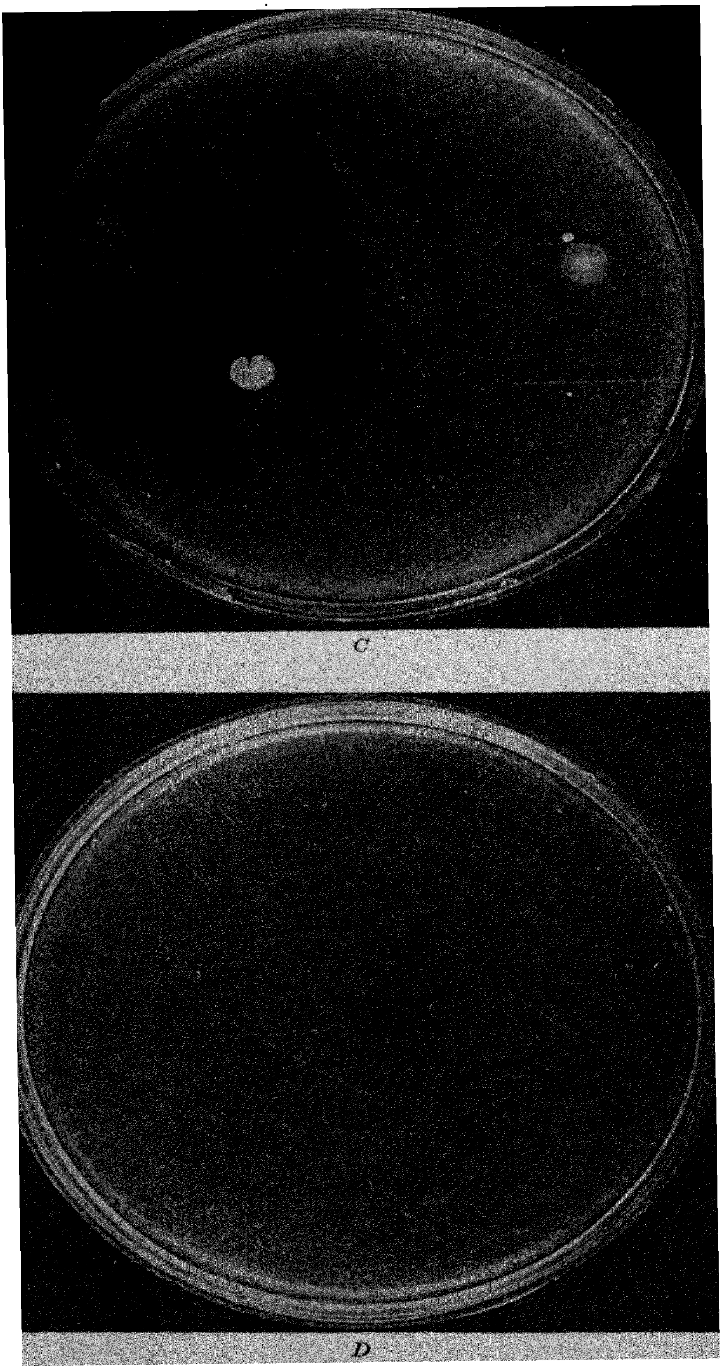


FIG. 105.—For descriptive legend see p. 149.

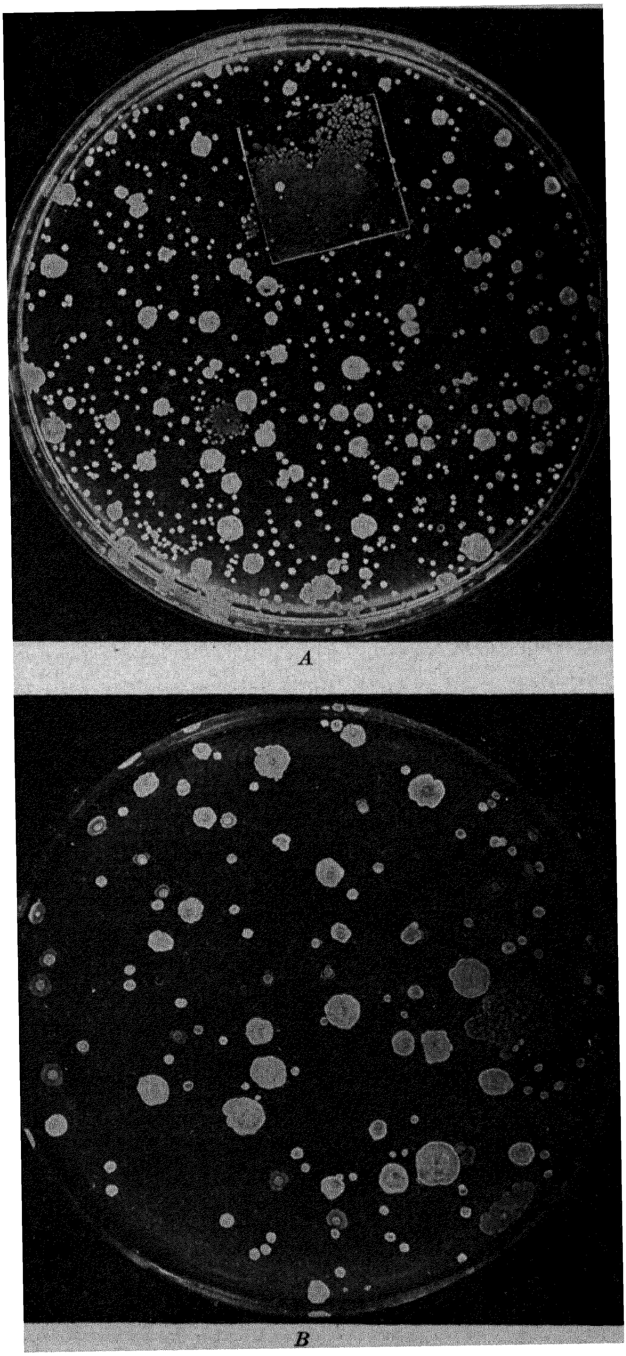
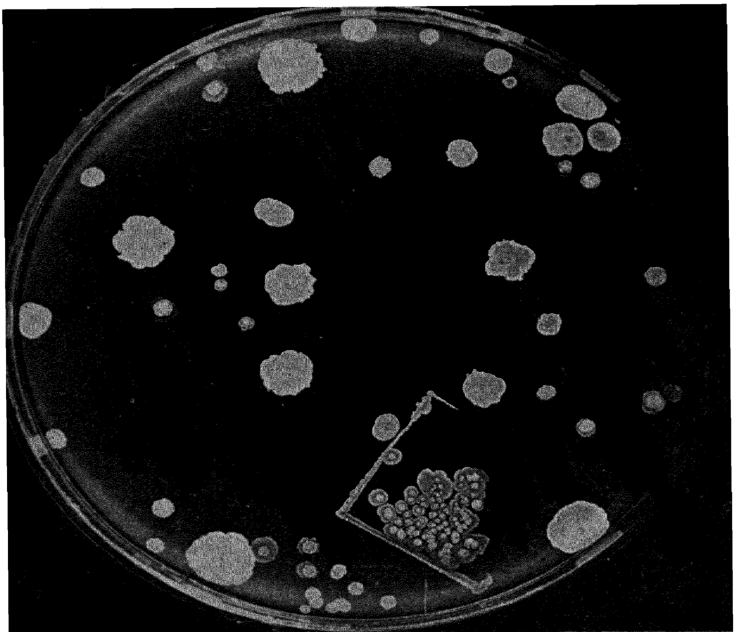


FIG. 106.—Effect of desiccation on *Bacillus subtilis*. *A*, control; *B*, dried 48 hr.; *C*, dried 96 hr.; *D*, dried 144 hr.



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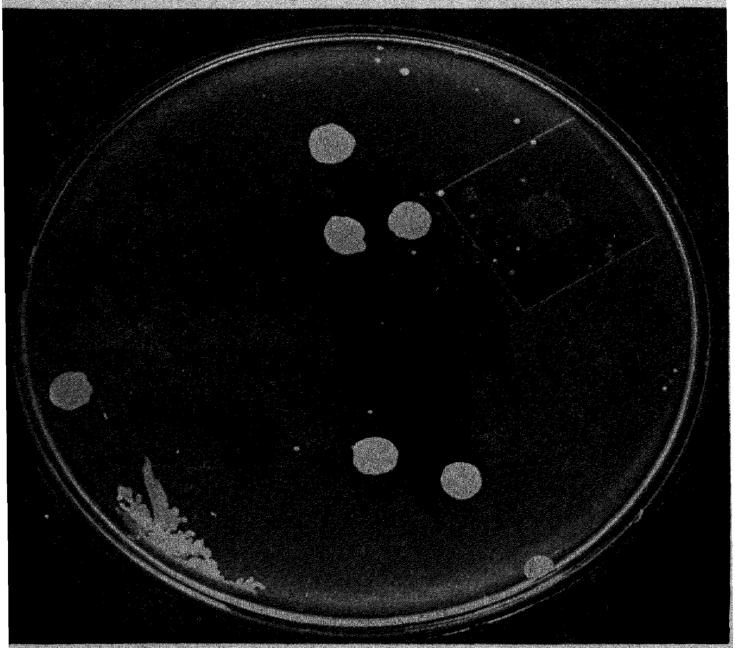

D

FIG. 106.—For descriptive legend see p. 151.

Ordinarily, it is necessary to transfer recently isolated strains of *Neisseria gonorrhoeae* at least twice a week and older strains about once a week in order that the organisms remain viable. Hac (1940) made a study of the various methods for the preservation of over 400 strains of this organism. Those preserved by sterile paraffin oil seals remained viable after transfer at 6- and 12-month intervals.

This method possesses distinct advantages over those already mentioned: (1) the method is inexpensive; (2) the cultures do not evaporate; (3) dissociation is prevented; (4) transfers are easily made to fresh medium; (5) no special apparatus is necessary, such as desiccators, vacuum pumps, etc.; and (6) the cultures are protected against mold contaminations.

Regardless of which method in this group is followed, no one of them will preserve bacteria for indefinite periods of time. All organisms slowly decrease in viability and finally die unless transfers are prepared to fresh medium at definite intervals.

Preservation by Rapid Desiccation.—Kitasato (1889) observed that the organism of cholera, *Vibrio comma*, survived longer when dried in a desiccator than when dried in air. Since that time many workers have desiccator-dried other organisms and have reported similar results. The effect of desiccation at 37°C. on survival of *Escherichia coli* and *Bacillus subtilis* is shown in Figs. 105 and 106.

Shackell (1909) recommended freezing as a preliminary step to rapid desiccation. The cultures may be frozen in salt-ice mixture or by means of solid carbon dioxide (dry ice), and then desiccated in vacuum over sulfuric acid as a desiccant. This method with its many modifications, especially those recommended by Swift (1937) and Flosdorf and Mudd (1935, 1938) is widely used at the present time for the preservation of bacteria, sera, viruses, enzymes, and other biological products.

Advantages to the methods in this group are that organisms may be preserved for longer periods of time than is possible by the methods listed in the first group. Also, the organisms do not show any appreciable changes in their morphological, biochemical, and immunological properties.

EFFECT OF HYDROGEN-ION CONCENTRATION

It is well recognized that the influence of acidity and alkalinity of culture media is of prime importance for the successful cultivation of bacteria. Some organisms grow best in acid environments; others grow best in alkaline media; still others grow best in neutral substrates. There exists for every organism an optimum concentration of hydrogen ions in which it will grow best. The hydrogen-ion concentrations above and below which an organism fails to grow are known as the minimum

and maximum pH values, respectively. The values are true only if the other environmental factors are kept constant. Variations in such factors as composition of the medium, temperature of incubation, osmotic pressure of the medium, etc., even though slight, will produce changes in the minimum, the optimum, and the maximum hydrogen-ion values of the organism. The distance from the minimum to the maximum is known as the pH range of an organism.

In 1912 Michaelis and Marcora noted that *E. coli* fermented lactose with the production of acids until a pH of about 5.0 was reached. This acidity was sufficient to prevent further growth of the organisms. They stated that the final hydrogen-ion concentration of cultures of *E. coli* is a physiological constant. Similar results have been reported by others working with different organisms. It may be concluded that any fermentative organism is limited in its acid fermentation upon reaching a rather definite hydrogen-ion concentration. The final degree of acidity will vary depending upon the species. These findings have been very important in differentiating closely related groups of organisms (see page 429).

EFFECT OF SURFACE TENSION

Surface tension may be defined as that property, due to molecular forces, which exists in the surface film of all liquids and tends to bring the contained volume into a form having the least superficial area.

Molecules attract their neighbors and are attracted by them. A molecule situated in a liquid will be in equilibrium by virtue of the equal attractions on all sides. On the other hand, a molecule situated on the surface of a liquid will have equal horizontal attractions but unequal vertical attractions. This results in an unbalanced attraction toward the interior of the liquid. This resultant force reaches a maximum at the surface and the mass of liquid behaves as if surrounded by an elastic membrane, tending to compress the liquid into the smallest possible volume.

The composition of the surface layer of a culture medium, inoculated with an organism, may differ appreciably from the composition of the same medium taken as a whole. The surface tension of media may, therefore, play an important role in the growth of an organism. This is especially true in the case of those organisms which have a tendency to grow on the surface of culture media in the form of a film or pellicle.

Organisms growing on the surface of a medium in the form of a pellicle were at one time regarded as strict aerobes. The surface layer, exposed to air, gave the organisms more oxygen than could be obtained from the deeper portions of the medium. It is true that the pellicle-forming bacteria are aerobic but not obligately aerobic. They are

capable of growth and multiplication under both aerobic and anaerobic conditions. Bacteria have a density slightly greater than that of the culture medium in which they are grown. If the pellicle produced by a pellicle-producing organism, such as *Bacillus subtilis,* is sedimented, the pellicle will not rise to the surface again but will remain at the bottom of the tube. A new pellicle will develop on the surface of the medium. It is obvious that the surface film is supported in this position by some force in the medium. This force is spoken of as surface tension.

The unit of force in the C.G.S. system of physical units is the dyne. It is such a force that under its influence a particle whose mass is 1 gm. would experience during each second an acceleration of 1 cm. per

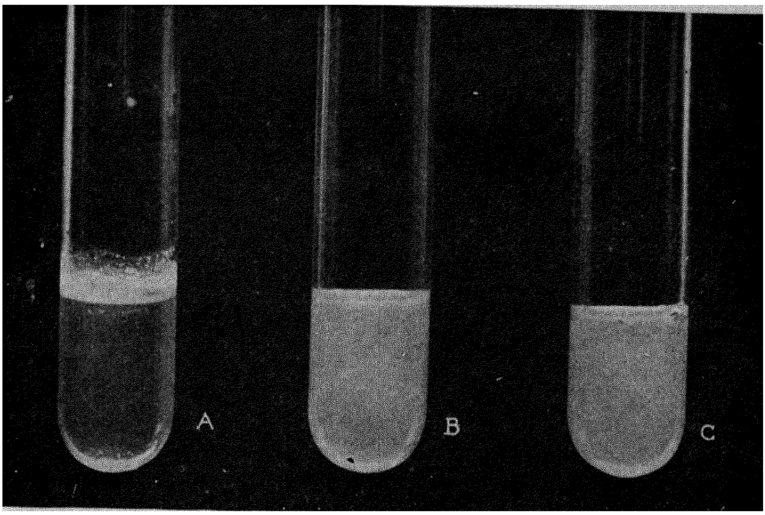

Fig. 107.—Effect of changes in the surface tension on the growth of *Bacillus subtilis.* *A*, growth in nutrient broth; *B* and *C*, growth in nutrient broth containing sodium ricinoleate.

second. The dyne is approximately the force exerted by a milligram weight under the influence of gravity.

The surface tension of the usual laboratory media varies between 57 and 63 dynes. The surface tension of pure water is 73 dynes. Some substances may be added to culture media to raise surface tension, among which may be mentioned charcoal. This substance is effective by virtue of its ability to remove some surface tension depressant from media. The growth of bacteria may, in some cases, raise the surface tension of the medium in which they are cultivated.

On the other hand many substances may be added to culture media to lower their surface tensions. Among these may be mentioned alcohol, saponin, and soaps. Soaps are probably more satisfactory for bacteriological media than any other group of compounds.

Larson, Cantwell, and Hartzell (1919) found that, if the surface tension of nutrient broth is depressed to some point below 40 dynes by means of soap and then inoculated with *B. subtilis*, the organism produced a diffuse growth rather than a pellicle on the surface. This same principle was found to apply to other pellicle-producing organisms. The formation of a pellicle apparently has nothing to do with the oxygen requirements of an organism but is dependent entirely upon the surface tension of the medium (Fig. 107).

Wetting is a function of surface tension. If bacteria are not wetted by the medium, they will grow on the surface in the form of a film; if the bacteria are wetted, they will not produce a pellicle but instead a uniform clouding of the medium. It has been shown that organisms growing on the surface of media contain a higher content of lipoidal substances than nonpellicle-producing organisms. The organism of tuberculosis has been found to contain as high as 40 per cent lipoidal substances in contrast to nonpellicle producers, which contain about 7 per cent.

Larson and Larson (1922) showed that organisms that ordinarily produce a uniform turbidity of a medium can be made to grow in the form of a pellicle if the lipoidal content can be increased. The fat content of *Staphylococcus aureus* is greatly increased if the organism is grown on a medium containing a carbohydrate or glycerol, neither of which is fermented. The organism produces a pellicle on the surface, which resembles very much the growth of the tubercle bacillus.

For an excellent summary of the effect of surface tension of the menstruum upon bacteria consult the article by Larson (1928).

ANTAGONISTIC ACTION OF IONS

Winslow and Falk (1923*a*) reported that *E. coli* maintains itself in distilled water at a pH of about 6.0 without material decrease in bacterial numbers for a period of nearly 24 hr. Increases in numbers may occur during the first few hours. At pH 5.0 the reduction is somewhat greater. The viability decreases as the solution is adjusted to more acid or alkaline conditions. This is shown in Table 7. A heavy line is drawn to indicate the range of time and pH most nearly corresponding to a one-third reduction in bacterial numbers.

On the other hand sodium chloride in a strength of 0.0145*M* exerts a distinctly favorable action on the viability of *E. coli*. Instead of a slight but definite decrease in numbers after 24 hr., as occurred in distilled water at pH 6.0, the bacteria maintain themselves in undiminished numbers. Above a concentration of 0.0145*M* NaCl the percentage of living organisms decreases with increasing concentration of the salt.

Similar results are obtained if calcium chloride is substituted for the sodium chloride. The organisms maintain themselves better in the

presence of this salt than in distilled water alone. Here again a pH of 6.0 appears to be the most favorable hydrogen-ion concentration for the growth or maintenance of *E. coli.* The most favorable concentration appears to be a solution of 0.00145*M* strength.

TABLE 7.—VIABILITY OF *Escherichia coli* IN DISTILLED WATER

Hours	Per cent alive at pH						
	4.0	5.0	6.0	6.5	7.0	7.5	8.0
1	87	88	84	92	68	77	79
3	39	71	74	66	54	24	52
6	4	48	64	30	24	8	12
9	1	68	82	7	17	5	12
24	0	6	77	2	23	3	10
Number of experiments	2	2	4	2	10	2	4

In another communication Winslow and Falk (1923*b*) reported that solutions of NaCl of 0.725*M* strength and over and solutions of $CaCl_2$ of 0.435*M* strength and over exhibit distinctly toxic actions on *E. coli* at all hydrogen-ion concentrations. However, in a solution containing a mixture of these two salts in appropriate proportions an antagonistic action is manifested, which tends to protect the bacteria against the toxic action that would be exerted by each salt if present alone (Table 8). This phenomenon is spoken of as the antagonistic action of ions.

TABLE 8.—VIABILITY OF *Escherichia coli* IN SOLUTIONS OF NACL AND $CACL_2$, SINGLY AND IN COMBINATIONS

Total isotonic concentration*	Total molar concentration	Percentage of bacteria alive after 9 hr. in			
		Pure NaCl	Pure $CaCl_2$	NaCl + $CaCl_2$	Ratio, Na/Ca
0	0	89	89	89	
1	0 145	82	22		
2	0 290	. .	. . .	41	1:1
3	0.435	55	0+	28	2:1
4	0.580	. .	. .	40	3:1
5	0.725	46	0+	117	4:1
6	0.870	33	. . .	30	5:1

* 1 tonicity = 0.145*M*.

In conclusion it may be stated that the toxic effects exerted by salts may be of two distinct kinds. Very high concentrations of salts appear to exert a toxic effect that is apparent at all reactions and is additive when

sodium and calcium chlorides are mixed. At a lower concentration (0.145M) calcium chloride exerts a different influence, manifest only in alkaline solutions and due to an inhibition of the power of the bacteria to reduce the alkalinity of the solution in which they are suspended. It is this latter type of toxic influence which is antagonized by sodium chloride. In alkaline solution the mixture of these salts in the proportion of 4 parts NaCl to 1 part $CaCl_2$ is more favorable to viability than even distilled water.

A practical application of this phenomenon is the use of a physiological salt solution developed by Ringer before ionic antagonism was clearly understood. Ringer showed that when a beating heart was perfused with a 0.75 per cent solution of sodium chloride, pulsation stopped completely. On the addition of 0.0125 per cent calcium chloride to the solution the heart beat was restored but not in a normal manner. On the further addition of 0.01 per cent potassium chloride and a small amount of alkali, such as sodium bicarbonate to adjust the pH of the solution, the heart beat became normal. This solution is known as Ringer's solution. Other physiological salt solutions of this type are Tyrode's and Locke's solutions.

OLIGODYNAMIC ACTION OF HEAVY METALS

Naegeli (1893) noted that silver in very high dilutions produced a toxic action on certain organisms. He found that 1 part of silver in 100,000,000 parts of water killed algae belonging to the genus *Spirogyra*. Naegeli believed that silver in such a high dilution could not produce a chemical action on living organisms. He, therefore, attributed the toxic effect of silver to an oligodynamic action. The word oligodynamic is compounded from the two Greek words, ὀλίγος, *oligo*, few, little, small, and δυναμικός, *dynamic*, powerful. It may be defined as the toxic effect produced on living organisms by heavy metals in exceedingly minute quantities.

It has been shown that other metals also exert a toxic action on organisms. Copper in a dilution of 1 part in 77,000,000 of water is toxic to certain algae. The spores of *Aspergillus niger* fail to germinate in the presence of 1 part of silver in 1,600,000 parts of water. Water distilled from a copper still is toxic to bacteria. This is due to the presence of traces of dissolved copper in the water. Water distilled from stills made of other heavy metals also exhibits this same phenomenon but to a lesser degree. This is not due to the greater toxicity of copper, as it is known that mercury is probably the most toxic metal, but to the fact that copper is more soluble in water than the other toxic metals. Therefore, metal stills should be avoided for the preparation of distilled water intended for biological use.

Lisbonne and Seigneurin (1936) studied the action of pure mercury on several species of bacteria suspended in distilled water, river water, stored water, and salt water. They employed 1 cc. of metallic mercury to each 1000 cc. of bacterial suspension. They concluded that (1) the efficiency of sterilization depended, on the one hand, upon the quantity of mercury employed and the surface utilized and, on the other hand, upon the nature and number of organisms; (2) a sterilized suspension of organisms separated from free mercury retained bactericidal properties for several hours, even after prolonged boiling. In a later communication Lisbonne and Seigneurin (1936) showed that mercury could be used for the destruction of *E. coli* in drinking water.

The use of silver has been recommended for the treatment of water, milk, vinegar, wine, cider, fruit juices, liquors, etc. Its greatest application appears to be in the treatment of water for drinking purposes. According to Gibbard (1937) three methods are generally followed commercially for the application of silver: (1) In one method water is exposed to silver deposited on sand, porcelain, and other solid materials. (2) In another method the silver is applied by electrolysis. (3) In still another method use is made of the difference in E.M.F. which exists between nickel and silver electrodes kept in the material at different temperatures. In all the methods the objective is the same, namely, to obtain a solution of the silver.

Demonstration of Oligodynamic Action.—Silver exerts a very marked bactericidal or oligodynamic action on bacteria. This may be easily demonstrated by placing a piece of metallic silver in a Petri dish and pouring over it melted agar, previously inoculated with an organism such as *E. coli*. After an incubation period of 24 hr. a clear zone will be seen immediately surrounding the silver metal. This is the oligodynamic zone (Figs. 108 and 109). Beyond this will be a narrower zone in which growth is stimulated. Minute amounts of metallic ions stimulate growth while greater concentrations produce an inhibitory action. Normal growth occurs in the remainder of the agar. The same result is produced if a piece of copper or copper coin is substituted for the silver.

Action of Silver on Bacteria.—Gibbard came to the following general conclusions regarding the action of silver on bacteria: (1) The width of the oligodynamic zone is increased by treating the silver with nitric acid and is decreased by careful cleaning of the metal. (2) Pure silver metal shows no bactericidal or oligodynamic action and its toxic properties are probably due to silver ions coming from silver oxide. If silver oxide is prevented from forming, no inhibitory action is noted. This may be shown by melting silver, allowing one portion of it to cool in hydrogen, and another portion to cool in air. The silver cooled in hydrogen shows no bactericidal action while the metal cooled in air exhibits a pronounced

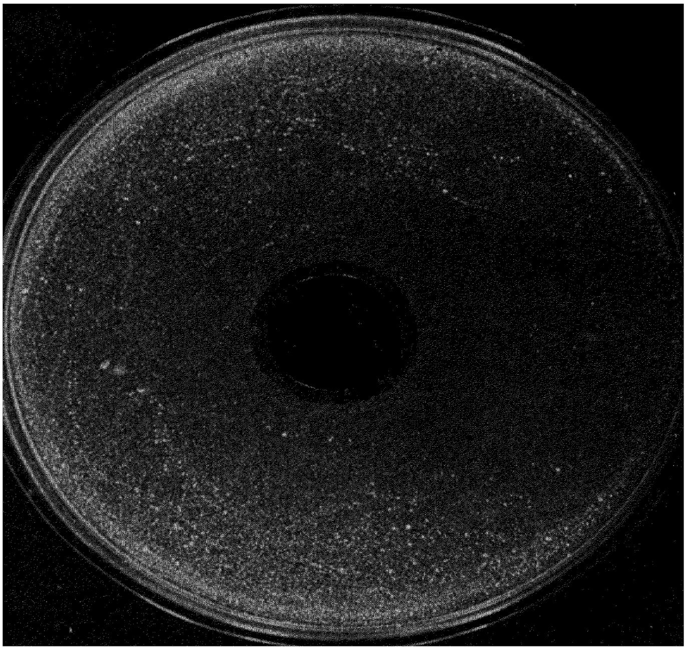

FIG. 108.—Oligodynamic action of silver on *Staphylococcus aureus.*

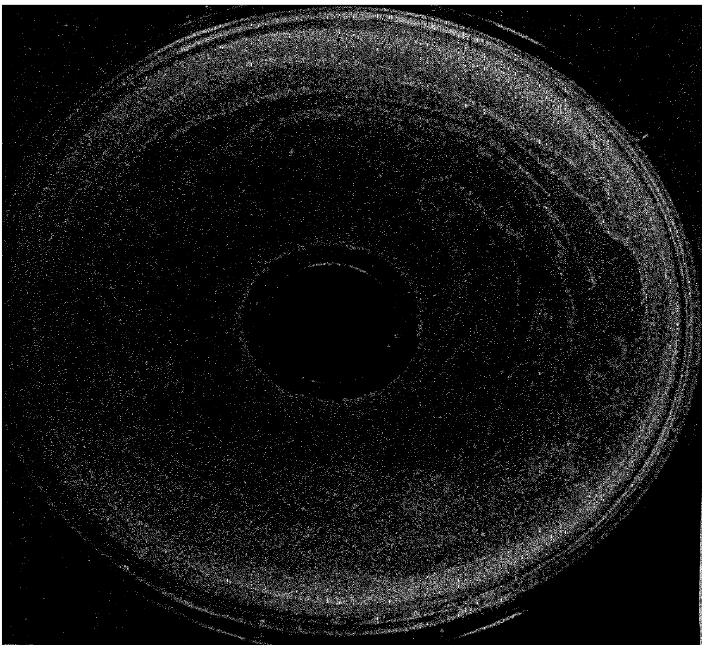

FIG. 109.—Oligodynamic action of silver on *Escherichia coli.*

inhibitory effect. (3) Silver nitrate, silver oxide, and electrically dispersed colloidal silver, when properly diluted to contain the same concentration of silver, all possess a similar bactericidal action. (4) The bactericidal properties of silver nitrate and silver oxide are greatly reduced in the presence of proteins. Silver proteinates are formed, removing the metal from solution.

Silver Poisoning.—There is still considerable discussion as to whether sufficient silver remains in treated water, foods, beverages, etc., to produce silver poisoning or argyria in man and animals. It is important to know how much of the silver ingested will be retained in the body. So far as is known there are no available data on this point. More work is required before this controversy can be definitely settled.

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CHAPTER IX

STERILIZATION

Sterilization may be defined as the complete destruction of all living microorganisms in, or removal from, materials by means of heat, filtration, or other physical or chemical methods.

Plugged test tubes, flasks, pipettes, bottles, etc., must be sterilized before use in order to destroy all living organisms adhering to the inner surfaces of the glassware. Likewise, all culture media must be sterilized previous to use to destroy all contaminating microorganisms present. Studies on single bacterial species or pure cultures could not be made if the glassware and culture media were contaminated with other kinds of organisms previous to use. When once sterilized, glassware may be kept in a sterile condition indefinitely if protected from outside contamination. The same applies to culture media if, in addition to sterility, evaporation can be prevented.

The usual procedures employed for the sterilization of glassware, media, cotton, etc., involve the use of heat. Three types of heat sterilizers are used in bacteriological laboratories for the destruction of living microorganisms. These are known as the hot-air sterilizer, the Arnold sterilizer, and the autoclave.

Hot-air Sterilizer.—This is a dry-air type of sterilizer (Fig. 110). It is constructed with three walls and two air spaces. The outer walls are covered with thick asbestos to reduce the radiation of heat. A burner manifold runs along both sides and rear between the outside and intermediate walls. Convection currents travel a complete circuit through the wall space and interior of the oven, and the products of combustion escape through an opening in the top.

The hot-air sterilizer is operated at a temperature of 160 to 180°C. (320 to 356°F.) for a period of 1½ hr. If the temperature goes above 180°C., there will be danger of the cotton stoppers charring. Therefore the thermometer must be watched closely at first until the sterilizer is regulated to the desired temperature.

The hot-air sterilizer is used for sterilizing all kinds of laboratory glassware, such as test tubes, pipettes, Petri dishes, flasks, etc. In addition it may be used to sterilize other laboratory materials and equipment which are not burned by the high temperature of the sterilizer. Under no conditions should the hot-air sterilizer be used to sterilize culture media as the liquids would boil to dryness.

Arnold Sterilizer.—It is well known that moist heat is more effective than dry heat in destroying microorganisms. This is believed to be due to the following reasons: (1) Moist heat has greater penetrating power. (2) Death of organisms by heat is believed to be due to a coagulation of the proteins of the protoplasm. An increase in the water content of the protoplasm causes the proteins to coagulate at a lower temperature.

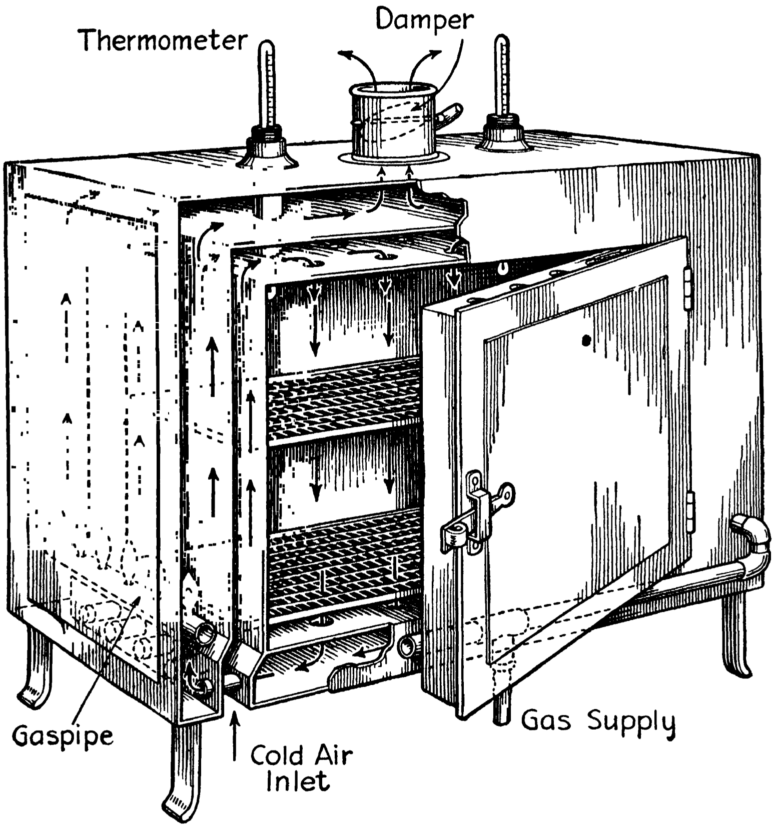


FIG. 110.—Hot-air sterilizer. (*From Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company.*)

The Arnold makes use of streaming steam as the sterilizing agent (Fig. 111). The sterilizer is characterized by a quick steaming base, which is automatically supplied with water from an open reservoir. The water passes from the open reservoir, through small apertures, into the steaming base to which the heat is applied. Since the base contains only a thin layer of water, steam is produced very rapidly. The steam rises through a funnel in the center of the apparatus and then passes into the sterilizing chamber.

Sterilization is effected by employing streaming steam at a temperature of approximately 100°C. (212°F.) for a period of 20 min. or longer on three consecutive days. The length of the heating period will depend upon the nature of the materials to be treated and the size of the container. Agar, for example, must first be completely melted before recording the beginning of the heating period.

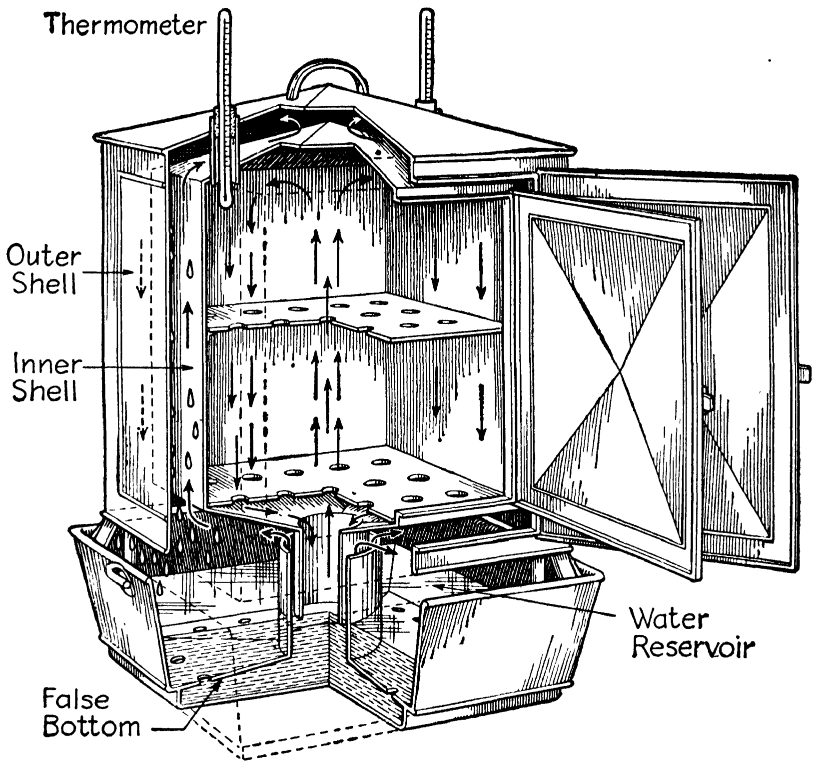


FIG. 111.—Arnold sterilizer. (*From Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company.*)

It must be remembered that a temperature of 100°C. for 20 min. or even for 1 hr. is not sufficient to destroy all spores. A much higher temperature is required to effect a complete sterilization in one operation over a relatively short period of time.

The principle underlying this method is that the first heating period kills all of the vegetative organisms (*i.e.*, not spores) present. After a lapse of 24 hr. in a favorable medium and at a warm temperature, the spores, if present, will germinate and pass into the vegetative cells. The second heating will again destroy all vegetative cells. It sometimes happens that all the spores do not pass into vegetative forms before

the second heating period. Therefore an additional 24-hr. period is allowed to lapse to make sure all spores have germinated into the less resistant vegetative forms.

It can be readily seen that unless the spores germinate the method will fail to sterilize. Failure may be due to the following causes: (1) The medium may be unsuited for the germination of the spores that may be present. Distilled water, for example, is not a favorable environment for the growth of organisms. It will not, therefore, permit spores to

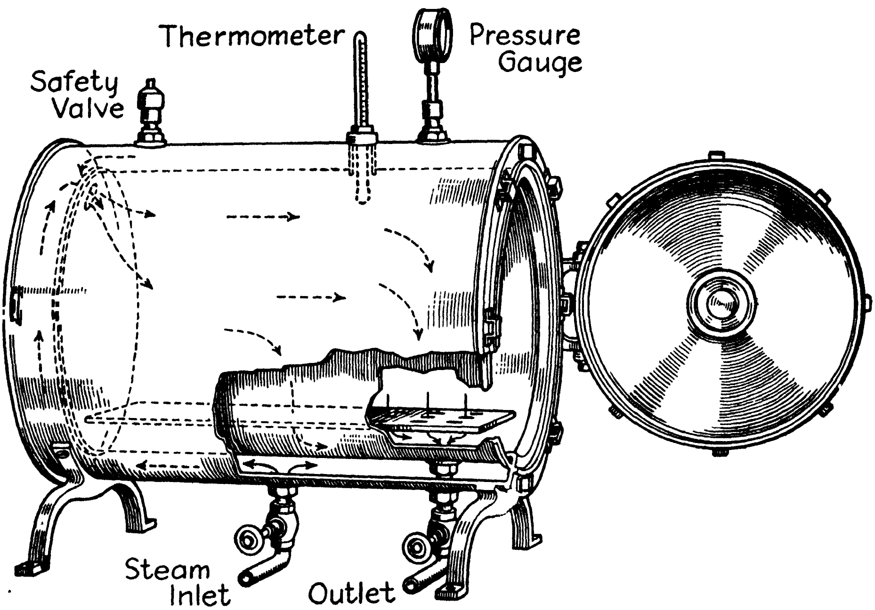


FIG. 112.—Autoclave sterilizer. (*From Belding and Marston, "A Textbook of Medical Bacteriology," D. Appleton-Century Company.*)

germinate into vegetative cells. (2) Anaerobic bacteria may be present which will not germinate in a medium in contact with atmospheric oxygen.

The Arnold is used principally to sterilize gelatin, milk, and carbohydrate media. Higher temperatures or longer single exposures in the Arnold may hydrolyze or decompose carbohydrates and prevent gelatin from solidifying. Obviously such media would then be unsatisfactory for use.

Autoclave.—The autoclave is a cylindrical metal vessel having double walls around all parts except the front (Fig. 112). It is built to withstand a steam pressure of at least 30 lb. per square inch.

The principle of the method is that water boils at about 100°C., depending upon the vapor pressure of the atmosphere. If the atmospheric pressure is increased, the temperature will be likewise increased.

Therefore, if the steam pressure is increased inside of the closed vessel to 15 lb. per square inch (2 atm.) the temperature will rise to 121.6°C. Table 9 will show the relationship between pressure and temperature:

TABLE 9

Pressure, lb. per sq. in.	Corresponding temperature	
	°C.	°F.
5	107.7	227
10	115 5	240
15	121 6	250
20	126.6	260
25	130 5	267
30	134 4	274

The autoclave is usually operated at 15 lb. steam pressure for a period of 30 min., which corresponds to a temperature of 121.6°C. This temperature and pressure are sufficient to destroy both vegetative cells and spores in one operation.

Certain precautions must be observed in order to prevent sterilization failures. The most important single cause of failure to obtain sterilization is due to incomplete evacuation of air from the chamber. Observation of the pressure gauge alone is not sufficient. The proper degree of temperature must also be taken into consideration. The temperature figures given in the table are true only if all air has been evacuated from the sterilizing chamber.

The temperature of a mixture of steam and air at a given pressure is less than the temperature of pure steam alone. This means that even though the autoclave is kept at the desired pressure the temperature may not be sufficient to give complete sterilization. This is shown in Table 10 by Underwood (1937).

TABLE 10

Degree of air evacuation	Pressure, lb. per sq. in	Ultimate uniform temperature in chamber, °C.
Complete	15	121
Two-thirds	15	115
One-half	15	112
One-third	15	109
No air evacuated	15	100

Another important point to be mentioned is that the method is effective only if the steam can penetrate the materials that are to be sterilized. If the steam is prevented from penetrating the materials,

the method will be of doubtful value. For example, suppose it is necessary to sterilize some cotton, placed inside a bottle, at 15 lb. pressure for 30 min. If the bottle is closed with a rubber stopper, the steam cannot reach the cotton. The process will not be any more effective than a hot-air sterilizer kept at 121.6°C. for a period of 30 min. It has already been said that such a temperature for 30 min. is insufficient to destroy spores in a dry-air sterilizer. If, on the other hand, the mouth of the bottle is covered with one or two thicknesses of muslin, permitting the steam to penetrate, then the cotton will be sterilized.

The autoclave is used to sterilize the usual noncarbohydrate broths and agar media, discarded cultures, contaminated media, aprons, rubber tubing, rubber stoppers, etc. This is the type of sterilizer that is used commercially for . . . . . . canned foods.

For further reading consult the report by Hoyt, Chaney, and Cavell (1938).

STERILIZATION BY FILTRATION

Some solutions cannot be sterilized by heat without being greatly altered in their physical and chemical properties. Serum in culture media is easily coagulated by heat. If the serum content is high enough, the medium becomes changed from a liquid to a solid preparation. Certain physiological salt solutions containing the unstable compound sodium bicarbonate are ruined if heated. The bicarbonate easily loses carbon dioxide and is converted into the more alkaline sodium carbonate. Enzymes and bacterial toxins in solution are easily destroyed by heat. These are but a few examples of many that may be mentioned.

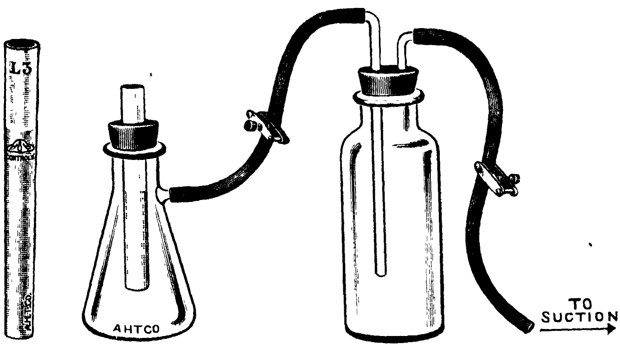
Preparations containing heat-sensitive compounds are best sterilized by the process of filtration. The types of bacterial filters employed include: porcelain filters, Berkefeld filters, Mandler filters, plaster of Paris filters, sintered glass filters, asbestos filters, and collodion membranes or ultrafilters.

Porcelain or Chamberland Filters.—Porcelain filters are hollow, unglazed cylinders and closed at one end. They are composed of hydrous aluminum silicate known as kaolin, with the addition of quartz sand; and heated to a temperature sufficiently low to avoid sintering. These filters are prepared in graduated degrees of porosity from L1 to L13. Cylinders having the finest (smallest) pores are marked L13 and those having the coarsest (largest) pores are designated L1. The finer the pores the slower will be the rate of filtration. The L1 and L2 cylinders are preliminary filters intended for the removal of coarse particles and large bacteria. The L3 filter is probably satisfactory for all types of bacterial filtrations. A satisfactory method for assembling a Chamberland filter is shown in Fig. 113.

Berkefeld Filters.—Kieselguhr is a deposit of fine, usually white siliceous powder composed chiefly or wholly of the remains of diatoms. It is also called diatomaceous earth and infusorial earth.

Berkefeld filters are manufactured in Germany. They are prepared by mixing carefully purified diatomaceous earth with asbestos and organic matter, pressing into cylinder form, and drying. The dried cylinders are heated in an oven to a temperature of about 1200°C. to bind the materials together. The burned cylinders are then shaped on machines into the desired shapes and sizes.

The cylinders are graded as W (dense), N (normal), and V (coarse), depending upon the relative sizes of the pores. The grading depends upon the rate of flow of pure filtered water under a certain constant pressure.



A *B*

FIG. 113.—*A*, Chamberland-Pasteur filter. *B*, filter assembled in a filter flask and ready for filtration. (*Courtesy of A. H. Thomas Company.*)

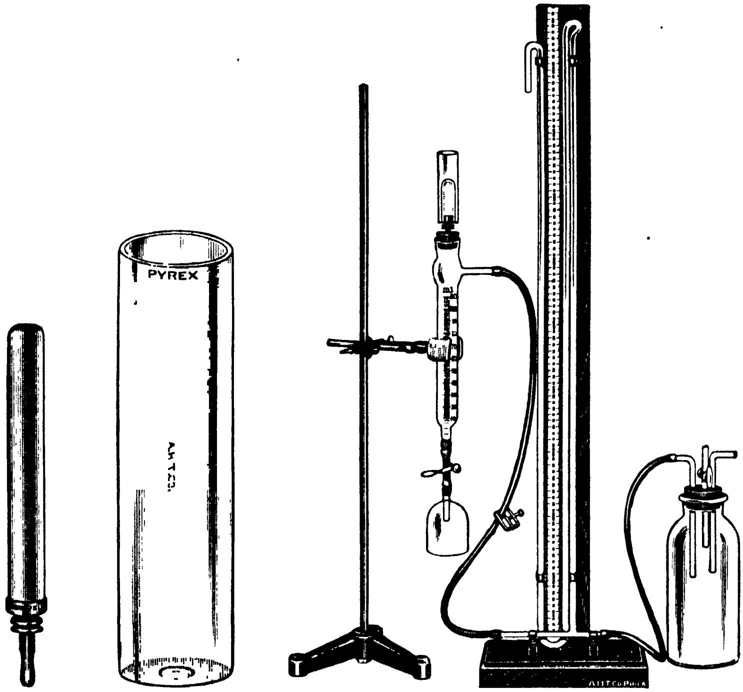
Mandler Filters.—These filters are similar to the Berkefeld type but are manufactured in this country. They are composed of 60 to 80 per cent diatomaceous earth, 10 to 30 per cent asbestos, and 10 to 15 per cent plaster of Paris. The proportions vary, depending upon the sizes of the pores desired. The ingredients are mixed with water, subjected to high pressure, and then baked in ovens to a temperature of 1800 to 3000°F. to bind the materials together.

The finished cylinders are tested by connecting a tube to the nipple of the filter, submerging in water, and passing compressed air to the inside. A gauge records the pressure when air bubbles first appear on the outside of the cylinder in the water. Each cylinder is marked with the air pressure obtained in actual test.

A convenient arrangement of apparatus for filtering liquids through a Mandler or Berkefeld filter, as recommended by Mudd (1927), is shown in Fig. 114. The reduced pressure is indicated by the manometer. The liquid to be filtered is poured into the mantle and the filtrate is received

into a graduated vessel from which it may be withdrawn aseptically. Filtration may be interrupted at any time by stopping the vacuum pump and opening the stopcock on the trap bottle to equalize the pressure.

Plaster of Paris Filters.—These filters are shaped like Berkefeld and Mandler filters but are composed chiefly of plaster of Paris or calcium sulfate with some calcium carbonate (chalk) and magnesium oxide. It is probable that the calcium sulfate acts as a binder for the calcium carbonate and that the latter substance is the active absorbing component of the filter (Kramer, 1927).



A B C

FIG. 114.—*A*, Berkefeld filter. *B*, glass mantle for use with either Berkefeld or Mandler filter. *C*, a convenient arrangement for assembling the filter for filtration. (*Courtesy of A. H. Thomas Company.*)

Plaster of Paris filters have the advantage over the other types of filters already discussed in that they may be easily prepared in the laboratory from cheap materials. They are attached to filter flasks and operated in the same manner as Berkefeld and Mandler cylinders.

A simple method for their preparation is shown in Fig. 115. A rubber stopper *A*, with a single perforation, is fitted into one end of a piece of heavy glass tubing *B*. Through the hole in the stopper is inserted a piece of glass tubing *C* containing a close-fitting glass rod *D*. A paste

is next prepared, composed of 75 gm. of plaster of Paris (containing 2.5 per cent calcium carbonate), 25 gm. of magnesium oxide, and 75 gm. of water. The paste *E* is poured into the glass cylinder and allowed to set. When the paste has set, the glass rod *D* is withdrawn, leaving a hole in the center of the filter for the filtrate to pass through.

Sintered Glass Filters.—Filters of this type are prepared by sintering finely pulverized glass into disk form in a suitable mold (Morton and Czarnetzky, 1937). The pulverized glass is heated to a temperature just high enough to cause the particles to become a coherent solid mass, without thoroughly melting, leaving the disk porous. The disk is then carefully fused into a glass funnel and the whole assembled into a filter flask by means of a rubber stopper. The assembled filter is shown in Fig. 116.

Asbestos Filters.—The best-known filter employing asbestos as the filtering medium is the Seitz filter. This is shown in Fig. 117. The asbestos is pressed together into thin disks and tightly clamped between two smooth metal rims by means of three screw clamps. The liquid to be filtered is poured into the metal apparatus, in which the asbestos disk is clamped, and the liquid drawn through by means of vacuum. The filtering disks are capable of effectively retaining bacteria and other particulate matter.

At the end of the filtering operation the asbestos disk is removed and discarded. A new one is inserted and the filter is again ready for use. This feature makes the Seitz filter very convenient to use since no preliminary cleaning is necessary.

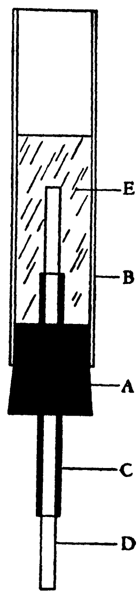


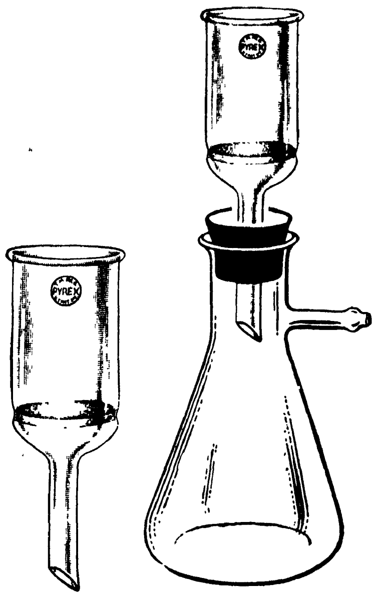
Fig. 115. Plaster of Paris filter.

Ultrafilters.—Ultrafiltration generally means the removal of colloidal particles from their solvents and from crystalloids by means of jelly filters, which are known as ultrafilters (Bechhold, 1926). The early jelly filters were composed of gelatin and silicic acid, but these have now been displaced by collodion in membrane and sac form, or collodion deposited in a porous supporting structure. The supporting structure may be filter paper in sheet and thimble form, unglazed porcelain dishes and crucibles, Buchner funnels, filter cylinders, etc.

Collodion.—Several types of collodions are employed for ultrafiltration. The earliest type is prepared by dissolving pyroxylin or soluble gun cotton in a mixture of 1 part of alcohol and 3 parts of ether. A more popular type is prepared by dissolving pyroxylin in glacial acetic acid. Pore size may be controlled by increasing or decreasing the pyroxylin content or by adding various liquids such as ethylene glycol and glycerol to alcohol-ether collodion. More recently Elford (1931) recommended

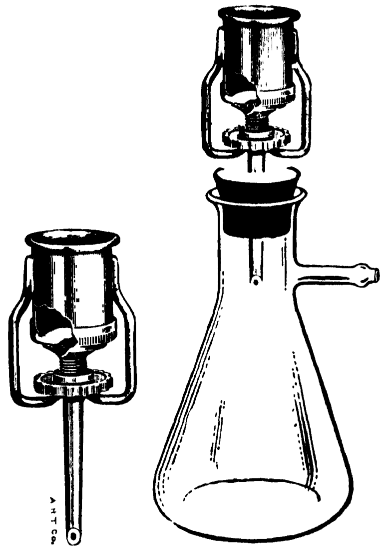
a new type of collodion for the preparation of a graded series of filters which he termed Gradocol membranes. The filters are prepared by incorporating a definite amount of amyl alcohol with an alcohol-ether collodion and then adding graded amounts of water or acetic acid to increase or decrease the permeability of the filters. Since membranes prepared by this procedure are quite strong, it is not necessary to deposit the collodion in a porous supporting structure.

Sacs may be prepared by pouring the collodion into a test tube or beaker, inverting and twirling continuously so that the excess drips out,



A B

FIG. 116.—*A*, sintered glass filter. *B*, filter assembled in a filter flask and ready for filtration.



A B

FIG. 117.—*A*, Seitz filter with asbestos disk in place. *B*, filter assembled in a filter flask and ready for filtration. (*Courtesy of A. H. Thomas Company.*)

until a thin even coating is formed. The tube or beaker is then plunged into cold water to jell the collodion. After most of the solvent has been washed away the sac can be loosened from the glass mold and removed. Collodion membranes in sheet form may be prepared by cutting the sacs at right angles at the closed end and then lengthwise.

Membranes employing filter paper as the porous supporting structure are usually prepared with acetic collodion. Pore size of the membranes depends upon the strength of the collodion. A strong collodion gives finer pores than a weak collodion. It is a simple matter to prepare a graded series of filters. The paper foundation gives the membranes a

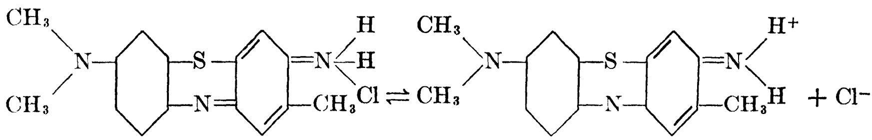
strength that, under certain conditions, will withstand a pressure of 20 atm. or more.

Electrical Charge of Filters.—The filtration of solutions of enzymes, toxins, immune bodies, viruses, etc., usually results in a loss of some of the active material. If the active material is present in very low concentration, it may be completely removed from solution.

Filters composed of porcelain (Chamberland), diatomaceous earth (Berkefeld, Mandler), and asbestos (Seitz) consist chiefly of metal silicates.

The metal (Mg^{++}, Al^{+++}, Ca^{++}, etc.) cations or positive ions are more soluble than the silicate anions or negative ions and show a greater tendency to pass into solution. When a liquid is filtered, positively charged particles will react with the negative silicate ions and negatively charged particles will react with the positive metal ions. Since the metal ions are soluble, they will react with the negatively charged particles and pass through the pores of the filter into the filtrate. On the other hand the insoluble silicate ions will react with positively charged particles and remain fixed to the walls of the pores of the filter.

Adsorption of compounds from solution can be very effectively demonstrated by means of basic and acid dyes such as toluidine blue and picric acid respectively. Toluidine blue is a basic dye belonging to the thiazine group and has the same chromophore as methylene blue and thionine. It ionizes as follows:

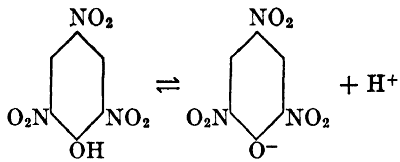


The cation is blue and the chlorine anion is colorless. When a solution of this dye is filtered through one of the silicate filters, the blue cations will react with the negative silicate ions and remain fixed to the pores of the filter. The chloride anions will combine with the metal cations of the silicates to form soluble metal chlorides and pass into the filtrate. If more than sufficient dye is present in the solution to react with all of the silicate ions in the filter pores, the excess will pass through, imparting a blue color to the filtrate. If, on the other hand, the amount of toluidine blue is insufficient to take care of all of the silicate ions, the dye will be completely removed from the solution and the filtrate will be colorless. The reaction is reversible, however, since the passage of distilled water through the filter saturated with dye results in a blue color in the filtrate.

If a solution of an acid dye, such as picric acid, is used instead of the basic toluidine blue, the results will be quite different. The dye will

not be adsorbed by the filter material but will pass through the pores into the filtrate.

Picric acid is trinitrophenol, an acid dye, having the nitro group as the chromophore. It ionizes as follows:



In this case the cation is colorless and the anion is yellow. When a solution of this dye is filtered through one of the silicate filters, not a trace of it will be adsorbed because an exchange of ions results in the formation of soluble picrates, which pass through in the filtrate.

Sintered glass filters, like the silicate types, carry a negative electrical charge. Michaelis (1925) found that collodion filters were nonionogenic but that they also carried a negative charge. Their negative charge was believed to be due to the adsorption of negative ions. Elford (1933) found that proteins in solutions adjusted to different pH values by hydrochloric acid and sodium hydroxide were most strongly adsorbed in the isoelectric zone (page 62). On the other hand proteins in solutions buffered with $M/15$ phosphate instead of adjusting the pH with hydrochloric acid and sodium hydroxide are adsorbed on the acid side of the isoelectric zone. The negatively charged collodion now preferentially adsorbs the positively charged proteins. He concluded that the effect is probably associated with some specific influence of the phosphate ion.

Plaster of Paris filters, on the other hand, carry a positive electrical charge. They are capable of producing insoluble compounds with anions, or negative ions, which remain adsorbed to the walls of the filter pores.

Amphoteric Nature of Proteins and Amino Acids.—An important characteristic of proteins and amino acids is that they contain both acidic (COOH) and basic (NH_2) groups. In acid solutions the compounds act as bases; in basic solutions they act as acids. Representing the formula of an amino acid as $R{\cdot}CHNH_2{\cdot}COOH$ the reactions with acids and bases are as follows:

With an acid:

$$\begin{array}{c} \mathrm{R} \\ | \\ \mathrm{H{-}C{-}NH_2} \\ | \\ \mathrm{COOH} \end{array} + \mathrm{HCl} \rightarrow \begin{array}{c} \mathrm{R} \\ | \\ \mathrm{H{-}C{-}NH_3Cl} \\ | \\ \mathrm{COOH} \end{array}$$

On ionization this gives

$$\begin{array}{c} R \\ | \\ H—C—NH_3Cl \\ | \\ COOH \end{array} \rightleftharpoons \begin{array}{c} R \\ | \\ H—C—NH_3^+ \\ | \\ COOH \end{array} + Cl^-$$

The acid reacts with the basic amino group. The amino acid molecule has a positive charge and, therefore, behaves as a base.

With a base:

$$\begin{array}{c} R \\ | \\ H—C—NH_2 \\ | \\ COOH \end{array} + NaOH \rightarrow \begin{array}{c} R \\ | \\ H—C—NH_2 \\ | \\ COONa \end{array} + H_2O$$

On ionization this gives

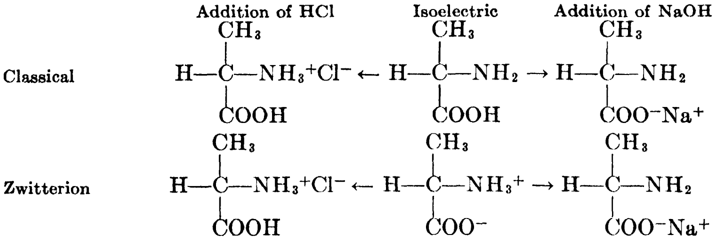
$$\begin{array}{c} R \\ | \\ H—C—NH_2 \\ | \\ COONa \end{array} \rightleftharpoons \begin{array}{c} R \\ | \\ H—C—NH_2 \\ | \\ COO^- \end{array} + Na^+$$

The base reacts with the acid carboxyl group. The amino acid molecule has a negative electrical charge and, therefore, behaves as an acid. Compounds of this nature that are capable of reacting with both acids and bases are said to be amphoteric (from the Greek, meaning both).

Isoelectric Point.—According to the classical theory, amphoteric compounds are supposed to dissociate into ions on either side of a pH point known as the isoelectric point. The isoelectric point has been defined as that point at which the ionization of the amphoteric electrolyte (ampholyte) is at a minimum expressed in pH. Opposed to this concept is the more recently developed view known as the "zwitterion" hypothesis. The difference in the two theories is indicated in the following formulas for isoelectric alanine:

$$\begin{array}{c} CH_3 \\ | \\ H—C—NH_2 \\ | \\ COOH \\ \text{I. Classical} \end{array} \qquad \begin{array}{c} CH_3 \\ | \\ H—C—NH_3^+ \\ | \\ COO^- \\ \text{II. Zwitterion} \end{array}$$

Formula I represents a molecule that is not dissociated either as an acid or as a base. The neutrality of the molecule is assumed to be due to the absence of dissociation. Formula II is also neutral but the neutrality is assumed to be due to complete ionization of the acid and basic groups. Regardless of which theory is correct, results obtained on the addition of an acid or a base are the same in both cases.



The isoelectric point of a protein is not necessarily the neutral point (pH7.0). As a matter of fact most proteins that have been studied have isoelectric points on the acid side of neutrality. The isoelectric points of a few of the common proteins are as follows:

	Isoelectric Point
Casein (milk protein)	pH4.7
Egg albumin	pH4.6
Gelatin	pH4.7
Hemoglobin	pH6.8

A knowledge of the isoelectric points is of considerable value in the filtration of solutions containing proteins, amino acids, bacterial toxins, enzymes, viruses, antitoxins, etc. If a solution is acid with respect to its isoelectric point, the active constituent will behave as a base and possess a positive electrical charge. The filtration of such a solution through a silicate filter, which has a negative charge, will result in the complete or partial adsorption of the active constituent on the walls of the filter pores. To avoid this it would be necessary to use a filter, such as plaster of Paris, that possesses a positive charge or to change the reaction of the solution to be filtered.

The adjustment of a solution to correspond to the acid or basic side of the isoelectric point can be carried out only provided the change in pH will not result in a destruction of the active material. After filtration, the pH of the filtrate should be readjusted to correspond to the optimum pH range of the active component. If the active material is very sensitive to slight changes in pH, a filter having an appropriate electrical charge should be selected instead.

Cleaning Filters.—All filters are intended to be used only once and then cleaned, or discarded and a new one employed. Collodion membranes and plaster of Paris filters are easily prepared in the laboratory and the Seitz asbestos disks can be purchased. Porcelain, diatomaceous earth, and sintered glass filters are too expensive to be used only once and then discarded, but they are easily cleaned in the laboratory.

Porcelain filters are cleaned by placing them in a muffle furnace and raising the temperature to a red heat. This burns the organic matter in the pores and restores the filters to their original condition.

Filters of the Berkefeld and Mandler types are cleaned by placing the cylinders in a special metal holder connected to the faucet. The flow of water is reversed by passing through the cylinder from within outward. This should be continued until all foreign matter has been washed away from the filter pores. Albuminous or similar materials remaining in the pores of the filters are likely to be coagulated by heat during the process of sterilization with the result that the filters will be clogged. Filters in this condition are useless for further work.

Clogged filters may be cleaned in various ways but probably most conveniently by continuous suction of full-strength Clorox solution for from 5 to 15 mins. (Vaisberg, 1938). This treatment quickly dissolves the coagulated material and again restores the usefulness of the filter. Thorough washing is necessary to remove the last traces of Clorox.

Sintered glass filters may be cleaned by treating with concentrated sulfuric acid containing sodium nitrate. The strong acid quickly oxidizes and dissolves the organic matter. Thorough washing is necessary to remove the last traces of acid.

Sterilization of Filters.—The types of filters already discussed are first sterilized before they may be employed. The filters should be assembled in their appropriate holders, wrapped in paper, and autoclaved. Dry heat cannot be used because of the destruction of rubber fittings for the filter flasks. Also, the collodion membranes would probably be burned by the high temperature of the dry-air sterilizer.

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technique, combining low tissue toxicity with high germicidal potency against *Staphylococcus aureus* (Gram-positive) and *Eberthella typhosa* (Gram-negative).

For further reading concerning other methods and germicides in general consult the articles by Garrod (1935); Thaysen (1938); Salle, Shechmeister, and McOmie (1940); Rose and Miller (1939, 1940); Miller and Rose (1939); Welch and Hunter (1940); and the monograph by McCulloch (1936).

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CHAPTER XI

NUTRITION OF BACTERIA

Culture media (singular, medium) are solid, semisolid, and liquid nutrient preparations employed for the cultivation of microorganisms. They are artificial environments prepared to simulate natural conditions as closely as possible.

The strict autotrophic organisms cannot use organic matter and are even harmed by its presence. These organisms are cultivated in preparations composed of pure inorganic salts only. They are able to synthesize complex compounds composing their protoplasm from simple inorganic salts. Because of this fact they are independent of vegetable or animal life. The strict heterotrophic organisms cannot synthesize their complex protoplasm from simple inorganic salts b [illegible]plex organic compounds, such as amino acids, coenzymes, or vitamins, for growth. Still other organisms show characteristics intermediate between the two, being able to utilize both inorganic and organic compounds. This group comprises the great majority of the bacteria that have been studied and classified. At one end of the scale the organisms exhibit complete independence; at the other end they show complete parasitism. Fildes (1934) advanced the theory that parasitism involved the loss of enzymes essential for the synthesis of bacterial protoplasm, making it necessary to add certain complex food substances to the culture medium.

The various ingredients employed for the preparation of the common laboratory media and their uses are as follows:

Water.—Water is absolutely necessary for the existence of all living cells. Distilled water is generally preferable to tap water because it is of definite composition. Uniform media cannot always be prepared from tap water. The calcium and magnesium in tap water react with the phosphates present in peptone, beef extract, and other ingredients of culture media to give insoluble calcium and magnesium phosphates. During the sterilization process such media throw down considerable precipitate, which usually proves to be objectionable.

Peptones.—Peptones are secondary hydrolytic products of proteins. Peptones for bacteriological purposes are produced by the action of certain proteolytic enzymes (usually trypsin) on native proteins. It is customary to consider peptones the last hydrolytic product that still

possesses true protein characteristics. In other words, the protein nature of the molecule ceases on further hydrolysis.

The commercial peptones are not the same as the peptones of the chemist who uses the term in its narrow, chemical sense. The commercial preparations employed in bacteriology are composed of proteoses or albumoses, peptones, peptids, and amino acids. The proportions vary considerably, depending upon the type of peptone desired. Some contain large amounts of proteoses with smaller amounts of peptones and amino acids; others contain small amounts of proteoses with larger amounts of peptones and amino acids; still others contain all the components in more or less well-balanced proportions. Some organisms prefer one type of peptone; others grow better in another type; etc.

Whole proteins such as casein and egg albumin are probably not attacked by bacteria, when used as the only source of nitrogen and carbon. They are indiffusible compounds and cannot enter the cell unless their molecules are broken up into smaller units. If a trace of commercial peptone is added to a protein, it will be readily attacked and utilized. The organisms utilize the amino acids of the peptone and, in so doing, elaborate the proteolytic enzymes required to attack the protein molecules. Amino acids readily pass through the cell walls of bacteria where they are used for structure and energy.

The most important function of peptone in culture media is to furnish an available source of nitrogen. Since amino acids are amphoteric compounds, peptone is also an excellent buffer.

Meat Extract.—Meat extract is prepared by boiling lean beef in water, removing the liquid by filtration, and concentrating in vacuo. It is a dark-colored, thick, pasty mass. Meat extract is prepared in such a manner that almost all food constituents are removed. For this reason it is not a good food in itself.

The constituents removed from muscle by boiling in water are known as extractives. The total amount of extractives, including both inorganic salts and organic matter, obtained from fresh muscle tissue by boiling in water amounts to about 2 per cent of the weight of the muscle. There are two classes of extractives obtained from meat: (1) the nitrogenous and (2) the nonnitrogenous. The nitrogenous extractives include creatine, xanthine, hypoxanthine, uric acid, adenylic acid, inosinic acid, carnosine, carnitine, glycocoll, urea, glutamine, β-alanine, etc. The nonnitrogenous extractives include glucose, hexosephosphate, lactic acid, succinic acid, inositol, fat, inorganic salts, etc.

The use of beef extract in culture media was introduced by Loeffler in 1881 and has been a routine procedure in bacteriology ever since. Meat extract is added to media to supply certain substances that stimulate bacterial activity. It contains enzyme exciters, which cause

accelerated growth of microorganisms. Recently McIlwain, Fildes, Gladstone, and Knight (1939) showed that glutamine, a constituent of beef extract, was a necessary nutrient for the growth of *Streptococcus pyogenes* (see page 215). More recently Williams (1941*a*) believed that β-alanine was present in beef extract in small quantities since it may arise by the hydrolysis of carnosine or in traces from pantothenic acid.

Gelatin.—Gelatin is a protein and is prepared by the hydrolysis of certain animal tissues, such as skin, ligaments, and bones, by treatment with boiling water. Gelatin is not soluble in cold water but swells and softens when immersed in it. It is quite soluble in boiling water. On cooling it solidifies to form a transparent jelly.

Gelatin is rarely added to media as a solidifying agent because (1) it is attacked and decomposed by many bacteria and (2) it melts at incubator temperature, 37°C. Gelatin is added to media principally to test the gelatinolytic powers of bacteria. Some organisms can liquefy it; others cannot. It is of importance in the identification and classification of bacteria.

Agar.—Koch (1881) recommended the use of gelatin as a solidifying agent to obtain pure cultures of organisms. The disadvantages to its use for this purpose have already been given. At the suggestion of Frau Hesse, Koch substituted agar for the gelatin to overcome these disadvantages.

Agar is the dried mucilaginous substance extracted from several species of *Gelidium* and closely related species growing chiefly along the coasts of Japan, China, Ceylon, and Malaya. Agar is a complex carbohydrate classified with the polysaccharides. It dissolves in water at a temperature of about 98°C. and does not solidify until the temperature drops to about 45°C.

Agar is attacked and liquefied by very few organisms. Recently Stanier (1941) described seven well-recognized marine species belonging to the genera *Vibrio*, *Pseudomonas*, and *Cytophaga* which are capable of liquefying agar.

Inorganic Requirements.—Although the forms of food required by microorganisms show considerable variation, it is believed that they must contain 10 known elements: (1) carbon, (2) hydrogen, (3) oxygen, (4) iron, (5) nitrogen, (6) calcium, (7) potassium, (8) magnesium, (9) sulfur, and (10) phosphorus.

The inorganic salts necessary for bacterial nutrition must depend to some extent upon the inorganic content of the cell. The ash content of bacteria, which is composed of inorganic compounds, has been shown to vary considerably both in amounts and proportions of the various elements. Variations in the ash content of *Escherichia coli*, as reported by Dawson (1919), are given in Table 28.

It is believed that the marked variations in salt content are due to the fact that some salts are exercising only a physical function; also, that the amount present in bacterial cells is dependent upon the inorganic content of the medium. On the other hand, some salts play an important part in the vital functions of protoplasm and enter into the more intimate structure of the cell. Guilleman and Larson (1922) showed that when *E. coli* was killed by heat about one-half of the inorganic salts diffused out of the cells, while the other half remained fixed inside of the cells. They suggested that the half that remained fixed inside of the cells was exercising some vital function. The fixed salts showed a high K/Na ratio in comparison to the free salts. Also, practically all of the iron remained inside of the cell, indicating that the element was necessary for protoplasmic activity.

TABLE 28.—VARIATIONS IN THE ASH CONTENT OF *Escherichia coli*

	Lowest, per cent	Highest, per cent
Water	60.2	79.6
Ash	2.11	7.83
P_2O_5	0.92	4.22
CaO	0.04	2 71

Sodium chloride is generally added to culture media to increase their osmotic pressures. A satisfactory medium is one that contains not only the necessary food substances in proper proportions but an osmotic pressure approaching an isotonic solution. Sodium chloride does not act as a buffer. Salts such as phosphates and carbonates do possess a strong buffering action and are frequently added to media for this purpose (see page 232).

Fermentable Compounds.—Fermentable substances are frequently added to culture media. Such compounds serve two functions: (1) they furnish readily available sources of energy provided the organisms elaborate the enzyme or enzymes necessary to ferment the compounds; and (2) fermentation reactions are of great help in identifying and classifying organisms.

The important fermentable substances added to culture media include

Monosaccharides:
- Pentoses: Arabinose, xylose, rhamnose.
- Hexoses: Glucose, levulose, mannose, galactose.

Disaccharides:
- Sucrose, lactose, maltose, trehalose, melibiose.

Trisaccharides:
- Raffinose, melezitose.

Polysaccharides:
  Starch, inulin, dextrin, glycogen, cellulose.
Alcohols:
  Trihydric: Glycerol.
  Pentahydric: Adonitol.
  Hexahydric: Mannitol, dulcitol, sorbitol.
Glucosides:
  Salicin, amygdalin.
Noncarbohydrate Compounds:
  Inositol.

NUTRITIONAL REQUIREMENTS

Culture media employed for the cultivation of bacteria may be divided into two groups on the basis of the character of the compounds making up their composition: (1) synthetic media, and (2) nonsynthetic media.

Synthetic Media.—The synthetic media are those in which the exact composition is known. They are made up largely of inorganic salts with some organic compound or compounds. The exact chemical make-up of all the components is known so that two batches of the same medium can be duplicated with a high degree of accuracy. Synthetic media are employed where it is desired to ascertain what effect an organism will have on certain organic and inorganic compounds. In other words, the nutritional requirements of bacteria can be accurately determined only by the use of synthetic culture media.

Nonsynthetic Media.—The nonsynthetic media are composed of compounds the exact composition of which is not known. Some of these compounds are peptones, beef extract, meat infusion, blood, and serum. It is practically impossible to prepare two identical lots of the same medium from different batches of the ingredients. Some of the more fastidious bacteria either do not grow or grow very poorly on synthetic media. However, these organisms are the exception rather than the rule.

Use of Nitrogen Compounds.—Koser and Rettger (1919) worked with a synthetic medium containing glycerol as the only source of carbon. To this medium were added various amino acids and other nitrogen-containing compounds, such as valine, glycocoll, asparagine, glutamic acid, phenylalanine, tyrosine, tryptophane, lysine, leucine, histidine, and urea. Of a total of 39 organisms studied, 21 were able to grow and multiply on such media. All of the amino acids employed were utilized as sources of nitrogen, with no marked differences in their availability. Asparagine, lysine, and the cyclic amino acid histidine apparently possessed no advantage over the monoamino acids. The compounds, urea, taurine, creatine, hypoxanthine, and uric acid were inferior to the

amino acids as available sources of nitrogen. Combinations of amino acids, or of amino acids and other nitrogenous compounds, were found to possess little advantage over any one of the amino acids. Dibasic ammonium phosphate yielded results only slightly different from those obtained from the amino acids. Compounds furnishing nitrogen as ammonia, whether amino acids or inorganic ammonium salts, were of equal value as an immediately available source of nitrogen.

Braun and Cahn-Bronner (1922*a*,*b*) employed ammonium lactate and other organic ammonium salts instead of ammonium phosphate and obtained essentially the same results. They found that all organisms that were capable of growing on ammonium lactate grew also if the ammonium salt was replaced by an amino acid. Their results confirmed those of previous workers, namely, that ammonia and amino nitrogen were interchangeable as sources of nitrogen for the nutrition of the organisms studied.

Fildes, Gladstone, and Knight (1933) reported results that showed that the amino acid tryptophane was necessary for the growth of *Eberthella typhosa.* They employed a basal medium containing sodium citrate, magnesium sulfate, phosphate buffer of pH7.4, and glucose. To this medium they added 14 amino acids and ammonium chloride as sources of nitrogen. The acids tested included alanine, glycine, leucine, valine, glutamic acid, asparagine, tyrosine, phenylalanine, proline, histidine, arginine, lysine, cystine, and tryptophane. The typhoid organism grew very well on this medium. By a process of elimination they found that the organism could derive its nitrogen requirement from a mixture of amino acids containing tryptophane, but ordinarily the organisms would not grow in the absence of this essential amino acid. Some strains of *E. typhosa*, which initially required tryptophane, could be trained to grow without it; others could not. From a nutritive standpoint, three varieties of the typhoid organism existed: (1) those which could grow with ammonia as the only source of nitrogen, (2) those which required tryptophane but could be trained to synthesize the compound, and (3) those which could not be trained to synthesize the compound. They concluded that tryptophane was probably an essential constituent of protoplasm and, if the organisms could not synthesize the compound, it must be added to the culture medium.

In a later communication Fildes and Knight (1933) placed organisms into three groups on the basis of their action toward tryptophane. The three groups are as follows:

1. This group includes those organisms which ordinarily cannot grow unless tryptophane is present in the medium. Some of the organisms in this group are, *Clostridium sporogenes, C. botulinum, C. tetani, Bacillus anthracis, Corynebacterium diphtheriae, Eberthella typhosa,* and *Staphylococcus aureus.*

2. This group includes those organisms which do not require tryptophane to be present in the culture medium. Some of the organisms in this group are the autotrophic bacteria, members of the genera *Escherichia* and *Aerobacter*, *Pseudomonas aeruginosa*, *Salmonella aertrycke*, and *Mycobacterium tuberculosis* var. *hominis*. These organisms have been shown to contain tryptophane in their protoplasms and are, therefore, able to synthesize the amino acid.

3. This group includes those organisms intermediate between Groups 1 and 2 in their requirements of tryptophane. The organisms are unable to synthesize tryptophane on first transference to a medium in which it is absent but they can be trained to do so by gradual elimination of the compound. Organisms that behave in this manner include *E. typhosa*, *C. diphtheriae*, *S. aureus*, and probably *M. tuberculosis* var. *hominis*.

Recently Burrows (1939*a*,*b*) obtained essentially the same results as reported by Fildes, Gladstone, and Knight and on the basis of further studies came to the following conclusions:

. . . the function of tryptophane in the growth of the strains of typhoid bacilli which apparently require it, is not that of supplying a molecular configuration that the organism is unable to synthesize. It would appear, rather, that it exerts a stimulatory effect on growth, a kind of "trigger" action. Not only is tryptophane not "essential" in the original sense, but it is actually synthesized by the bacteria during the growth process.

Contrary to usual belief, the typhoid organism produced indole in extremely small amounts. This would suggest that some of the biochemical differences between bacteria may be a matter of degree rather than of kind, quantitative rather than qualitative, and in view of this implication, evidence which suggests the acquiring of new physiological characters by bacteria should be subjected to rigorous test before drawing conclusions.

Since *E. typhosa* is known to require tryptophane, Fildes (1940) tested a number of related but less complex compounds for their ability to support growth of the organism. The compounds tested included indoleacrylic acid, indoleacetic acid, indolepropionic acid, indolecarboxylic acid, indolepyruvic acid, indole-ethylamine, indolealdehyde, skatole, and indole. Indole stimulated the growth of the organism and, since indolecarboxylic acid contained a trace of indole as an impurity, this accounted for the slight growth obtained in the presence of the compound. The other substances were found to be entirely inactive. This observation suggested that indole was a stage in the synthesis of tryptophane by the typhoid bacillus, according to the scheme: NH_3 or amino N $\rightarrow$ indole $\rightarrow$ tryptophane. If the scheme is correct it should be possible to find an organism that can synthesize tryptophane when supplied with any of the stages given above. Results showed that some bacteria were capable of synthesizing tryptophane from any of its precursors whereas others required the finished amino acid. Fildes

concluded that tryptophane was synthesized from ammonia in stages, one of which is indole or a closely related substance. Failure of an organism to grow was due to inability to synthesize tryptophane.

McIlwain, Fildes, Gladstone, and Knight (1939) found that glutamine was an essential nutrient for the growth of some strains of *Streptococcus pyogenes* but not necessary for others. Glutamine is present in beef extract and is probably widely distributed in the animal body. In later communications Fildes and Gladstone (1939), and McIlwain (1939) reported that glutamine was indispensable for the growth of many strains of *S. pyogenes* and other bacteria. A large number of analogues and derivatives of glutamine were incapable of replacing glutamine in supporting growth of hemolytic streptococci.

Use of Carbon Compounds.—Braun and Cahn-Bronner tested a large number of carbon compounds including formic, acetic, oxalic, lactic, succinic, malic, tartaric, and citric acids, glycerol, glucose, and arabinose. They found that glucose, glycerol, lactic, and citric acids were utilized more than any of the other carbon compounds when tested against *Salmonella schottmuelleri*, *S. enteritidis*, and *Eberthella typhosa*. Acetic and oxalic acids ranked next. Formic and probably tartaric acids were not available as sources of carbon. The amino acids ranked lower than the organic acids, carbohydrates, and glycerol from the standpoint of availability. den Dooren de Jong (1926) tested about 250 organic compounds for their availability as sources of carbon for a number of organisms. The compounds were added to a synthetic medium containing ammonia as the only source of nitrogen. His conclusions were similar to those of Braun and Cahn-Bronner. He found that carbohydrates and related compounds were most generally utilized; these were followed by malic, citric, succinic, and lactic acids; next came the fatty acids; and last the monohydric alcohols.

Braun and Cahn-Bronner found that anaerobic growth was entirely absent when *S. schottmuelleri* was inoculated into an inorganic medium containing ammonium lactate and glucose. Koser (1923) found the same to be true when the members of the *Escherichia* and *Aerobacter* groups were inoculated into media containing various organic acids as carbon sources. Citric acid and its salts were utilized by *Aerobacter aerogenes* but not by *Escherichia coli* (see page 430).

Formation of Lipoids.—The formation of lipoidal substances by bacteria is dependent upon the nature of the carbon compounds added to the medium. Stephenson and Whetham (1922) employed an inorganic medium containing ammonium salts to which were added lactic acid, acetic acid, lactic and acetic acids, glucose, and glucose and acetic acid. The media were inoculated with *Mycobacterium phlei*, an acid-fast organism. Their results are shown in Table 29. The addition of

acetate to the various media produced no increase in protein formation but a large increase in the synthesis of lipoid material. Increased concentrations of lactate and of glucose increased both protein and lipoid, especially the former. An organism that normally synthesizes sufficient lipoid material to become acid-fast can be made to grow acid-sensitive by omitting from the culture medium an appropriate carbon source.

In a similar type of experiment Larson and Larson (1922) found that those organisms which fermented glucose or glycerol were unable to utilize the compound for the synthesis of lipoid material. Since *Escherichia coli*, *Staphylococcus aureus*, and *Clostridium mucosum* fermented

TABLE 29.—EFFECT OF COMPOSITION OF MEDIUM ON FAT FORMATION

Organic constituents of medium	Period of maximum lipoid formation, days	Milligrams per 100 cc. of medium		Ratio, $\frac{\text{lipoid}}{\text{nitrogen}}$
		Nitrogen synthesized	Lipoid synthesized	
0.68% lactic acid	10	19	16	0 84
0.4% lactic acid..	11	26	20	0 78
1.2% lactic acid 0.4% acetic acid	7	20	35	1.78
1% glucose.	12	18	28	1 6
1% glucose 1% acetic acid	21	18	42	2 34
1% glucose....	15	15	19	1 3
2% glucose..	15	34	21	0 91

glucose, they did not synthesize additional lipoid. On the other hand, those organisms which did not ferment glucose or glycerol utilized the carbonaceous material for the synthesis of lipoids. *E. coli*, *S. albus*, and *Bacillus megatherium* were unable to ferment glycerol. These organisms showed a marked increase in fat formation over either the nutrient broth controls or the glucose broth.

Geiger and Anderson (1939) inoculated *Phytomonas tumefaciens* into two synthetic media, one containing glycerol and the other sucrose. The organisms grown on the glycerol-containing medium yielded only 2 per cent of total lipoidal material whereas those grown on the sucrose-containing medium gave 6 per cent of fatty substances. The nature of

the fatty material synthesized by the organisms on the two media also showed considerable difference. Their results are recorded in Table 30.

GROWTH FACTORS AND VITAMINS

Hopkins (1906) was probably the first to point out that compounds other than fat, protein, carbohydrate, minerals, water, and oxygen were necessary in human nutrition. This observation led to the discovery of the vitamins by Funk (1912). Wildiers (1901) reasoned that, since certain factors not known at that time were required in human nutrition, a comparable situation existed in the requirements of bacteria. Wildiers employed an inorganic medium containing cane sugar and ammonia as sources of carbon and nitrogen, respectively, and found that yeast cells failed to grow unless a certain number of the organisms were transferred to fresh medium. The addition of a boiled suspension of yeast produced the same growth-promoting effect on small inocula as the addition of an emulsion of living yeast. Wildiers named the growth-promoting factor bios.

It is now known that bios is a mixture of several of the water-soluble members of the vitamin B complex. The constituents of the vitamin B complex known to possess bios properties include thiamin (vitamin B_1), pantothenic acid, pyridoxin (vitamin B_6), biotin (vitamin H, bios II B, coenzyme R factor), and inositol.

It has been shown that some bacteria require the presence of a number of recognized growth factors and some still unrecognized, whereas

TABLE 30.—YIELD OF LIPOIDS FROM DRIED *Phytomonas tumefaciens*

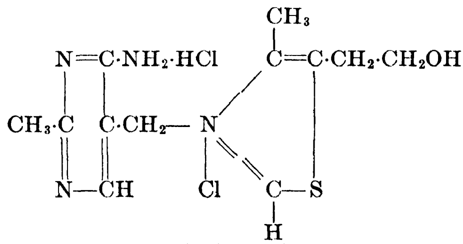
	Medium 1, grams	Medium 2, grams
Bacteria used for extraction . .	364.	276.
Alcohol-ether-soluble lipids	4.85	16 23
Chloroform-soluble lipids	2.46	0.63
Total phosphatide .	3.21	10.90
Total acetone-soluble fat	3 35	5.92
Ether-insoluble substance.	0 64	0.03

other organisms do not require the presence of these factors preformed. Those organisms which do not require the presence of growth factors in a preformed condition have been shown to be capable of synthesizing them. It is, therefore, erroneous to conclude that an accessory growth factor is not required by an organism simply because it is not present in the culture medium in which the organism is growing.

A large number of growth-promoting substances have been studied, some of which have been synthesized, and their chemical constitution

determined. Some are simple in structure and others are quite complex. These compounds are designated by various names, such as growth accessory substances, growth factors, growth determinants, vitamins, and coenzymes. Williams (1941*a*) coined the term "nutrilites" to include all organic substances, regardless of the nature of the agent, which in minute amounts are of importance in the nutrition of microorganisms.

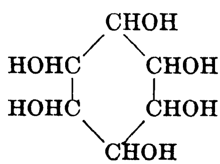
Thiamin Chloride (Vitamin B_1).—This is a component of bios and of the vitamin B complex. It is necessary for the growth of baker's or brewer's yeast (*Saccharomyces cerevisiae*). Chemically it is a pyrimidine-thiazole compound having the following structural formula:



Thiamin chloride

Tatum, Wood, and Peterson (1936) showed that thiamin played an important role in the growth of several species of the genus *Propionibacterium.* Later Silverman and Werkman (1938) verified their results. In a later communication Wood, Anderson, and Werkman (1938) worked with a larger number of species of *Propionibacterium* and found that several factors, including thiamin chloride, were required for growth. Some of the strains could be trained to grow vigorously without the nutrilite. Knight (1937) reported the necessity of this vitamin for the growth of *Staphylococcus aureus.*

Inositol.—This was the first pure substance isolated that was found to contribute to bios activity. The compound was isolated in pure form by Eastcott (1928) from tea. Inositol becomes a limiting growth factor only when other nutrilites are added. Inositol is present in both plant and animal cells but its exact physiological function is not clearly understood. It has the following structural formula:



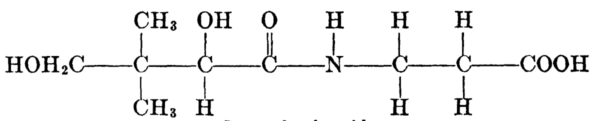
Inositol

Biotin.—This growth factor was first isolated by Kögl (1935). In a later communication he stated that the compound had the empirical

formula $C_{11}H_{18}O_3N_2S$. It is probably identical with coenzyme R and possibly with vitamin H.

Biotin stimulates the growth of yeast in extraordinarily small amounts. According to Kögl, an amount as small as 0.00004 μg. added to 2 cc. of culture caused a 100 per cent increase in growth. This is equivalent to 1 part in 50,000,000,000 parts of medium. The nutrilite has been shown to be present in animal and plant tissues. It is effective not only for yeasts but for various molds and bacteria. Snell and Williams (1939) found it to be the only growth accessory requirement for certain butyl alcohol-producing anaerobes. Porter and Pelczar (1940) attempted to cultivate *Staphylococcus aureus* in a chemically defined medium to which were added the growth factors, riboflavin, inositol, pimelic acid, glutamine, glutathione, pyridoxin, pantothenic acid cocarboxylase, cozymase, uracil, guanine, adenine, adenylic acid, and adenosine triphosphate but were unable to obtain growth. However, the addition of a minute amount of biotin to the medium produced vigorous growth of the organism indicating that this nutrilite was a limiting factor in the growth of *S. aureus*. Landy and Dicken (1941) cultured a large number of organisms in a synthetic medium without biotin and found that they were capable of synthesizing the growth factor to a greater or lesser degree. The evidence suggested that biotin might be of widespread importance in microbial nutrition.

Pantothenic Acid.—Williams and Bradway (1931) were the first to point out that a growth factor, which they designated pantothenic acid, was necessary for the nutrition of yeast. In a later communication Williams, Lyman, Goodyear, Truesdail, and Holaday (1933) stated that pantothenic acid was a growth determinant of universal biological importance, being present in the cells of higher plants and animals, in molds, bacteria, algae, yeasts, protozoa, etc. It was shown to consist of β-alanine united to a saturated dihydroxy acid by a peptid-like combination having the following formula:

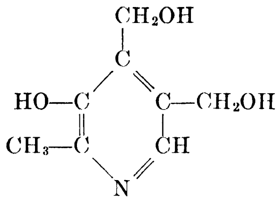


Pantothenic acid

Snell, Strong, and Peterson (1939) showed that a number of species of *Lactobacillus* and *Propionibacterium* required pantothenic acid and other growth factors for satisfactory growth. In a later communication Snell and Peterson (1940) found that pantothenic acid was a necessary factor for vigorous growth of *Lactobacillus helveticus*. Pelczar and Porter (1940*b*) reported the necessity of the factor for the growth of *Proteus morganii*. Berkman, Saunders, and Koser (1940) found that pantothenic

acid was a limiting factor for the growth of several species of *Pasteurella*. Rane and Subbarow (1940) reported that the factor was also necessary for the growth of several types of *Diplococcus pneumoniae*.

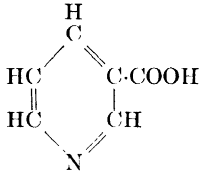
Pyridoxin (Vitamin B_6).—This substance was first recognized as a vitamin by György (1935). Keresztesy and Stevens (1938) and Lepkovsky (1938), working independently, isolated the vitamin in crystalline form. Harris and Folkers (1939) synthesized the vitamin and showed it to be 1-methyl-2-hydroxy-3,4-dihydroxy-methyl pyrimidine having the following structural formula:



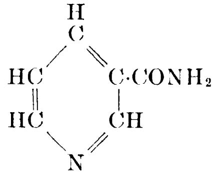
Pyridoxin

Schultz, Atkin, and Frey (1939) and Eakin and Williams (1939) found that the vitamin caused an increased growth of yeast. Robbins and Schmidt (1939) showed that the nutrilite stimulated the growth of excised tomato roots. The necessity of pyridoxin for some lactic acid bacteria was shown by Möller (1938) and more recently by Snell and Peterson (1940). McIlwain (1940) found that pyridoxin, together with other growth factors, was necessary for the nutrition of *Streptococcus pyogenes*.

Nicotinic Acid, Nicotinamide, and Coenzymes I and II.—Nicotinic acid was first prepared in 1867, but the nutritional importance of the compound was not recognized until 1937, by Elvehjem, Madden, Strong, and Woolley. The structural formulas for nicotinic acid and its amide are as follows:



Nicotinic acid



Nicotinamide

Knight (1937) added nicotinic acid and nicotinamide to a basal amino acid medium and found that both factors failed to permit growth of *Staphylococcus aureus*. When other factors were added to the medium, in addition to the nicotinic acid compounds, vigorous growth occurred. This indicated that the staphylococcus growth factor is complex and that only one component of it can be replaced by nicotinic acid or its more

active amide. In a later communication Knight (1938) reported the results of tests on a large number of pyridine derivatives and concluded that nicotinic acid and nicotinamide were highly specific in the growth requirement of *S. aureus.* Only very limited departures from these two structures were permissible if growth activity was to be maintained. Similar results were reported by Landy (1938). Rane and Subbarow (1938) concluded from their results that glutathione, thiochrome, flavin, nicotinic acid, betaine, and glucosamine, in the presence of a calcium-alcoholic precipitate of a highly purified liver extract, were required for vigorous growth of a strain of *Streptococcus pyogenes* in a deficient medium. The presence of all the factors provided almost optimum conditions. Omissions of one or more factors decreased the amount of growth.

Dorfman, Koser, Reames, Swingle, and Saunders (1939) and Dorfman, Koser, Horwitt, Berkman, and Saunders (1940) tested 32 strains of *Shigella dysenteriae* in a basal synthetic medium alone and with the addition of nicotinic acid, nicotinamide, and coenzyme I or II. Many of the strains were unable to grow in the synthetic medium but developed readily upon the addition of a small amount of any one of the foregoing compounds. Nicotinamide was found to be at least 10 times more potent than nicotinic acid in 24-hr. cultures, but the difference was less marked in older cultures. Several other essential growth factors were synthesized by the organisms.

Pelczar and Porter (1940*a*) tested 189 strains of *Proteus vulgaris* and related species for their ability to grow in a simple basal medium to which was added nicotinic acid or one of 13 other related pyridine compounds. The results indicated that the same pyridine compounds that were found to be biologically active for *S. aureus* were likewise active for the majority of the *Proteus* cultures investigated. However, the same medium was unable to support growth of 37 strains of *P. morganii.* In a later report Pelczar and Porter (1940*b*) tested a number of growth factors alone and in various combinations. These included thiamin chloride, pyridoxin, riboflavin, β-alanine, pimelic acid, nicotinic acid, inositol, cocarboxylase, glutamine, and coenzyne I. They reported that *P. morganii* failed to grow on a medium containing all the above factors, but the addition of a small amount of pantothenic acid produced vigorous growth. The investigators concluded that pantothenic acid and nicotinic acid were the essential growth factors for *P. morganii.* Berkman, Saunders, and Koser (1940) in their studies on 17 cultures of the genus *Pasteurella* found nicotinamide, together with other factors, a growth essential for these organisms. Snell and Peterson (1940) reported the necessity of nicotinic acid for the growth of *Lactobacillus casei.*

Pfeiffer (1893) reported that the organism *Hemophilus influenzae*, which was isolated from the sputum of patients suffering from influenza, would not grow in a broth medium unless blood was added. Thjötta (1921), Thjötta and Avery (1921*a*,*b*), and Fildes (1921, 1922) found that the same organism required the presence of two factors which they named the *V* and *X* factors.

Both factors are present in blood. The *V* factor is also present in many plant extracts and in a large number of bacteria. It is thermolabile, being destroyed in 15 min. at 90°C., is very sensitive to alkali but not to acid, diffuses through parchment membranes, and is not readily destroyed by atmospheric oxygen. The *X* factor is thermostable, resisting a temperature of 120°C. for 45 min. It is present in potato and in some bacteria.

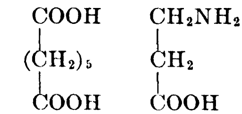
H. influenzae is unable to grow on culture media supplied with only the *X* factor. However, it will grow on such media in association with an organism, such as *S. aureus*, which is capable of producing the *V* factor. The characteristic arrangement of colonies of *H. influenzae* in such an association is sometimes referred to as the satellite phenomenon. The hemophilic organisms grow as satellites in isolated colonies at some distance from the colonies of *S. aureus*.

The organism *Hemophilus parainfluenzae* requires only the *V* factor for growth. Lwoff and Lwoff (1937*a*,*b*) isolated the *V* factor from yeast and added the extract to a culture of the organism. They noted that *V* activity paralleled the coenzyme I concentration of the yeast extract. This substance was found to replace the *V* factor in extremely low concentrations. Coenzymes I and II are very similar chemically but are not, as a rule, interchangeable. As growth factors, however, coenzyme II can replace coenzyme I. Since one or the other factor must be supplied before growth occurs, they are considered to be true vitamins. Chemically coenzyme I is diphosphopyridine nucleotide and coenzyme II is triphosphopyridine nucleotide (see page 280).

It has been stated that the *X* factor supplies some hematin compound but its exact nature is still uncertain.

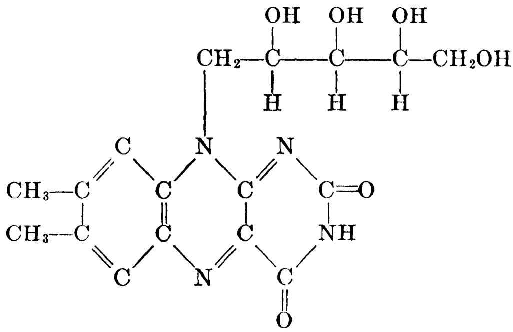
Pimelic Acid and β-Alanine.—Mueller (1935) and Mueller and Kapnick (1935) showed that the diphtheria bacillus *Corynebacterium diphtheriae* produced a luxuriant growth in a medium composed entirely of amino acids to which were added a purified liver extract and a carbon source, such as glycerol and lactic acid, and suitable inorganic salts. Later Mueller (1937*a*,*b*) identified two of the constituents of the liver extract, essential for growth of the diphtheria bacillus, as pimelic acid and nicotinic acid. In a later report Mueller and Cohen (1937) found β-alanine to be a third growth-accessory substance present in liver extract responsible for the luxuriant growth of the diphtheria bacillus

in a synthetic medium. The structural formulas for pimelic acid and β-alanine are as follows:



Pimelic acid β-alanine

Riboflavin.—Other names for this factor are lactoflavin, vitamin B_2, and vitamin G. It is a *d*-ribose derivative of iso-alloxazine having the following formula:



d-riboflavin

Riboflavin has been shown to be necessary for the growth of many bacteria. Snell and Strong (1939) found that of 11 species of *Lactobacillus* investigated, 4 required the addition of the vitamin to the medium and 7 did not. Of the 7 species that did not require the presence of the vitamin in the medium, 4 were capable of synthesizing the compound. Street and Reeves (1940) cultivated the tubercle bacillus on a synthetic medium and found that the organism was capable of synthesizing the growth factor.

GROWTH PHASES IN A CULTURE

It is well known that the smaller an object the greater will be the ratio of the area to its weight. For example, an organism of average size, such as *Escherichia coli*, has a volume of 2×10^{-9} cu. mm. and a surface area of 10^{-5} sq. mm. If the specific gravity is taken as 1 (bacteria have a specific gravity slightly greater than 1), the weight of a single cell of *E. coli* will be 2×10^{-9} mg. The ratio

$$\frac{\text{Area } (10^{-5} \text{ sq. mm.})}{\text{Weight } (2 \times 10^{-9} \text{ mg.})} = 5000$$

A similar calculation for an average man becomes

$$\frac{\text{Area (2.4 sq. meters)}}{\text{Weight (100 kg.)}} = 0.024$$

Comparing the two results, it is seen that the ratio of area to weight is about 200,000 times greater for a bacterial cell than for a man.

Bacterial organisms absorb the necessary nutrients through their cell walls. Because of this fact, the greater the ratio of surface area to weight the greater will be the amount of food absorbed in proportion to size. This explains why bacteria are able to multiply at such a rapid rate and produce great changes in culture media in a short period of time. Under favorable conditions a single cell of *E. coli* divides into two about every 20 min. If this same rate is maintained, a single organism will give one billion new cells after a period of about 10 hr. However, this rate of multiplication is not maintained indefinitely owing to the exhaustion of the nutrients, to the accumulation of toxic metabolic waste

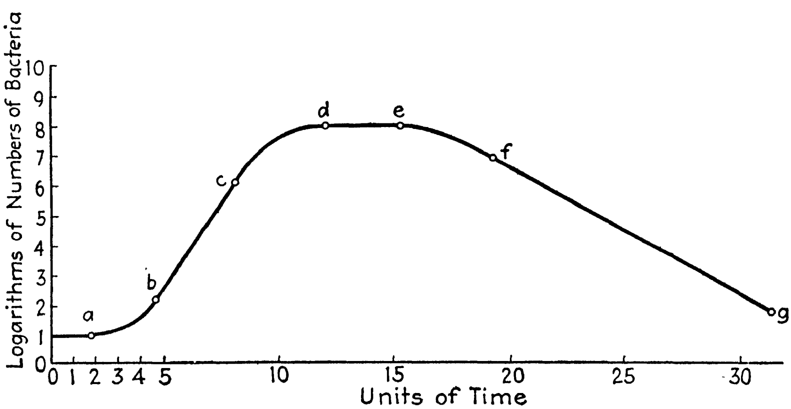


FIG. 121.—Growth phases in a culture. (*After Buchanan.*)

products, and to the fact that many of the cells die. The rate of death increases as the culture ages. The more vulnerable cells die first, leaving the resistant forms in the culture at the end of the incubation period.

When an organism is inoculated into a tube of medium, such as nutrient broth, multiplication does not take place in a regular manner but various growth phases may be distinguished, which are known as the life phases of a culture. Buchanan (1918) recognized seven distinct cultural phases which he designated as follows:

1. *Initial Stationary Phase.*—During this phase the number of bacteria remains constant. Plotting the results on graph paper gives a straight horizontal line (1*a*) in Fig. 121.

2. *Lag Phase or Phase of Positive Growth Acceleration.*—During this phase the rate of multiplication increases with time (*ab*).

3. *Logarithmic Growth Phase.*—During this phase the rate of multiplication remains constant (*bc*). This means that the generation time is the same throughout.

4. *Phase of Negative Growth Acceleration.*—During this phase the rate of multiplication decreases (*cd*). The average generation time increases. The organisms

continue to increase in numbers but at a slower rate than during the logarithmic growth phase.

5. *Maximum Stationary Phase.*—During this phase the number of living organisms remains constant, *i.e.*, the death rate equals the rate of increase (*de*).

6. *Phase of Accelerated Death.*—During this phase the numbers fall off with increasing rapidity (*ef*). The average rate of death per organism increases to a maximum.

7. *Logarithmic Death Phase.*—During this phase the rate of death is constant (*fg*).

Lag Period.—When bacteria are transferred from an old culture to a new medium, they exhibit a period of delayed multiplication or lag. Müller (1895) was probably the first to make this observation. He recognized three distinct phases in a culture, which he designated the lag, the logarithmic increase, and the slackened growth phases. Müller found that when cultures of differing ages were used for the inoculation of new medium, the generation times in the new cultures showed considerable differences. Transfers from a typhoid culture 2½ to 3 hr. old gave a generation time of 40 min. in the new medium; a culture 6¼ hr. old gave a generation time of 80 to 85 min.; and a culture 14 to 16 hr. old gave a generation time of 160 min.

Another characteristic of organisms is that they show considerable variation to harmful influences in the different growth phases. Reichenbach (1911) found that cultures in the lag and early logarithmic phases exhibited greater sensitivity to heat than those in the older phases of growth. Schultz and Ritz (1910) reported that a 20-min. culture of *Escherichia coli* was more resistant to heat than a 4-hr. culture. Then the resistance showed a steady rise as the culture aged. Sherman and Albus (1923) exposed *E. coli* to various unfavorable conditions and found that old cells were considerably more resistant than very young cells. Hegarty and Weeks (1940) found that young cells of a culture of *E. coli* were more susceptible to cold shock than old cells. Mature cells were not affected by either an initial cold shock or a prolonged holding at 0°C. They concluded that the sensitivity of young cells to cold appear to be related in some manner to cell division and to changes within the individual cell.

If a tube of fresh medium is inoculated from a culture in the logarithmic growth phase (*bc*, Fig. 121), the lag phase will be greatly reduced and in many cases completely eliminated. The organisms in the logarithmic growth phase are multiplying at the maximum rate and continue to do so when transferred to fresh medium. Buchanan (1928) concluded that transfer from any phase of a bacterial culture cycle to a new medium is followed by a continuance of the phase of the parent culture.

Factors Affecting Cell Size.—Henrici (1928) showed that organisms increased very markedly in size during the lag phase. He found that the average length of *Bacillus megatherium* was six times longer than the

inoculated organisms taken from an old culture. This increase generally manifested itself after 2 hr. and the maximum size was usually noted between 4 and 6 hr. During the lag phase the cells showed considerable fluctuation in form. On passing from the lag phase to the logarithmic death phase the organisms gradually decreased in size and exhibited a more constant cell form.

It is well known that when a parent culture is inoculated into a new medium an initially slow rate of increase in bacterial numbers occurs. Hershey (1939) showed quite conclusively that this slow rate of multiplication cannot be interpreted as indicating a period of lag in the sense of decreased viability and activity. What actually happens is that the rate of multiplication decreases but the individual cells become larger, giving a rapid increase in bacterial mass. Hershey inoculated new medium from 3- and 24-hr. parent cultures of *Escherichia coli* and measured the increase in bacterial mass by means of a photelometer. During the first 2 or 3 hr., new cultures inoculated from 3-hr. old parent cultures showed a slower multiplication rate than new cultures inoculated from 24-hr. parent cultures but the increase in protoplasmic growth remained the same. The cells from a young parent culture showed the same increase in cell mass as the cells from an old parent culture, even though they multiplied at a slower rate. The rate of increase in cell mass is nearly constant from the time growth first begins until the maximum population is reached.

The results suggest that conditions in fresh medium favor an increase in cell size but inhibit cell division with the result that a majority of the cells attain an abnormal size before fission occurs. Inoculation of a large number of cells into new medium tends to produce the reverse effect, *i.e.*, the average size is smaller and attained much sooner than with small inoculations. If cells are removed from a culture before their average maximum size is reached and transferred to fresh medium, the organisms attain a larger size and the critical point takes place later than in the case of the original culture. This would indicate that the size of the organisms is dependent upon the density of the bacterial culture. The concentration of the nutrients in the medium is another factor. The maximum size of the organisms in a dilute medium is smaller and the critical point is reached earlier. A dilute medium produces a poorer growth than a more concentrated preparation. This means that a more concentrated medium showing a heavy, crowded growth produces the same effect on cell size as a more dilute medium showing a light growth.

Effect of Carbon Dioxide on Growth.—Valley and Rettger (1927) showed that many bacteria that they studied grew better in the presence of an increased concentration of carbon dioxide. Walker (1932) found

that the length of the lag phase could be controlled by the concentration of carbon dioxide present. He noted that the multiplication of *Escherichia coli* could be indefinitely delayed by aeration of a culture with CO_2-free air. Reintroduction of CO_2 into the medium caused a rapid increase in bacterial numbers. He concluded that "the phenomenon of lag may be due largely, if not entirely, to the time it takes the culture to build up the CO_2 content of the medium or of the cells themselves to a value essential for growth." Others have come to a similar conclusion. There appears to be no doubt that the amount of carbon dioxide present in a new medium is an important factor in controlling the length of the lag phase but it is probably not the only factor involved.

Factors Affecting Rate of Reproduction.—The rate of multiplication of bacteria is increased by a rise in temperature. This continues until a certain maximum is reached, after which the rate decreases until death finally occurs. The generation times of *E. coli* at different temperatures of incubation are as follows:

°C.	Time	°C.	Time
10	14 hr., 20 min.	35	22 min.
15	120 min.	40	17½ min.
20	90 min.	45	20 min.
25	40 min.	47½	77 min.
30	29 min.		

Penfold and Norris (1912) found that the generation time of *Eberthella typhosa* in 1 per cent peptone solution at 37°C. was about 40 min. If the amount of peptone in the medium was less than 0.2 per cent, the generation time was almost inversely proportional to the concentration of peptone; if above 0.4 per cent, an increase in the amount was practically without effect on the growth rate of the organisms. The addition of 0.175 per cent of glucose to a medium containing only 0.1 per cent peptone lowered the generation time from 111 to 50 min. The addition of the same amount of glucose to a 1 per cent peptone solution reduced the generation time only from 39 to 34 min.

Kojima (1923) believed that the phases in a bacterial culture were due to environmental changes in the medium, such as alteration in pH, exhaustion of food supply, and accumulation of waste products. A glucose broth medium was used. After an incubation period of 48 hr., sodium hydroxide was added to the glucose broth culture to neutralize the high acidity. This caused the viable count to rise from 12,000 to 192,000,000 cells at the end of 60 hr., showing that the decrease in multiplication was due to the low pH. However, the 60-hr. count did not

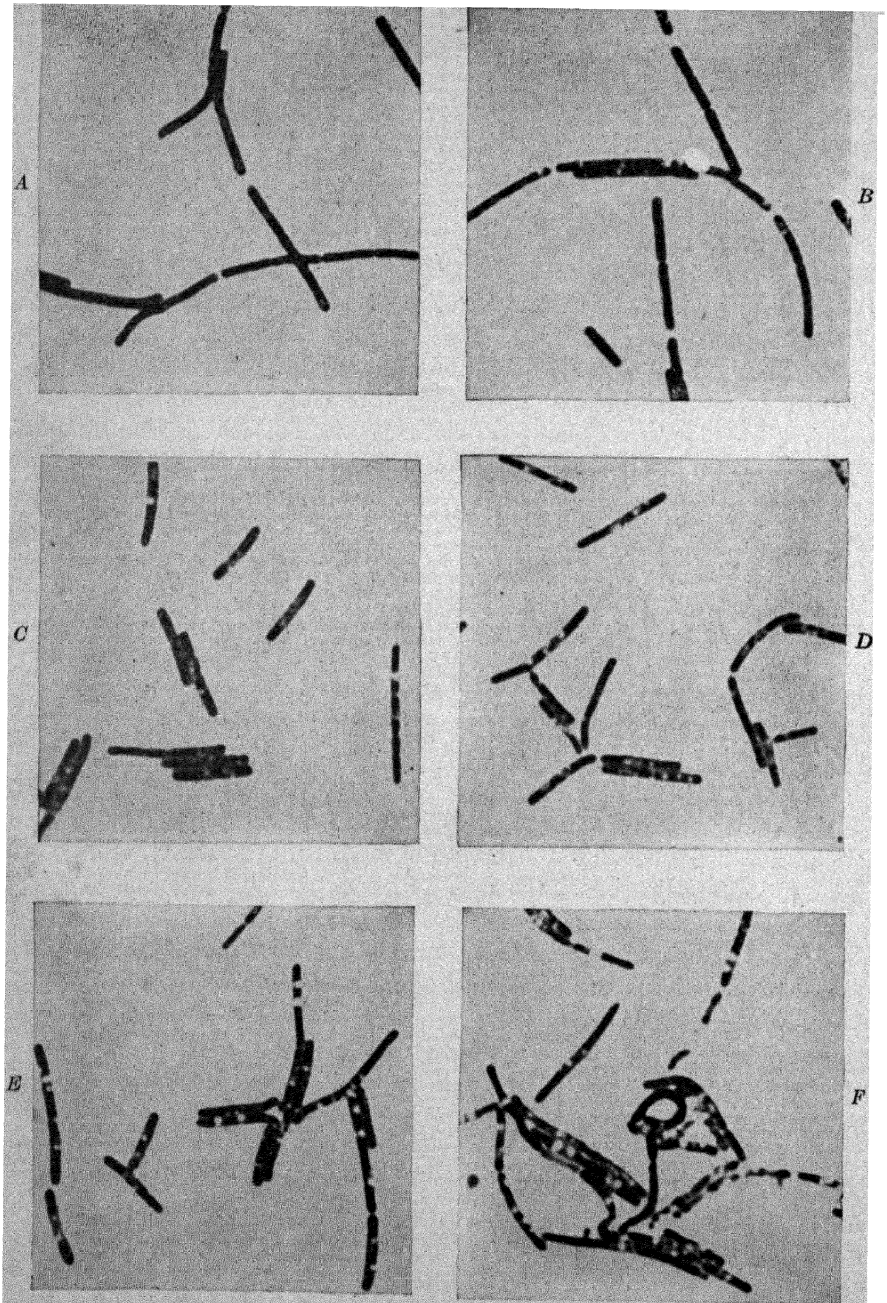


FIG. 122.—*Bacillus mycoides.* *A*, 6-hr. culture; *B*, 9-hr. culture; *C*, 18-hr. culture; *D*, 21-hr. culture; *E*, 24-hr. culture; *F*, 48-hr. culture. Note the development of granules with age.

reach the height attained in the same period of time as in the previous medium without glucose. This might indicate either an exhaustion of the nutrients or the presence of inhibitory waste products. To test the first hypothesis fresh nutrient broth was added, with the result that the count rose to 380,000,000 at the end of 60 hr. If glucose was also added, approximately the same count was obtained but the falling off occurred at a more rapid rate.

Effect of Age on Cell Morphology.—Under some environmental conditions bacteria show the presence of granules whereas under other conditions they do not. It has been observed that when cells are largest (2 to 4 hr. old) intracellular granules disappear and the protoplasm becomes more hyaline and stains more deeply. As the cells age and decrease in size they become increasingly more granular. Old cells are, in general very granular whereas young cells do not exhibit the presence of granules. The development of granules in cells of *Bacillus mycoides* with age is shown in Fig. 122. An exception to this rule is the organism *Corynebacterium diphtheriae,* the causative agent of diphtheria. This organism appears to be smaller in young than in old cultures and to exhibit the presence of granules in both young and old cells.

For further reading on bacterial nutrition consult the articles and monographs by Evans, Happold, and Handley (1939), Koser and Saunders (1938), Landy (1941), Mueller (1938, 1940), O'Kane (1941), Porter and Pelczar (1941), Stephenson (1939), Williams (1941*b,c*), and Winslow and Walker (1939).

HYDROGEN-ION CONCENTRATION OF CULTURE MEDIA

Culture media are adjusted to different degrees of acidity or alkalinity, depending upon the organisms to be cultivated. Some organisms grow best in media having an acid reaction; others require an alkaline reaction; still others prefer media that are neither acid nor alkaline but neutral in reaction. This last group includes the majority of organisms. It is necessary, therefore, to adjust the reaction of media to satisfy the requirements of the organisms being studied.

Two methods are employed for adjusting the reaction of culture media: (1) the determination of the actual numbers of free hydrogen ions and (2) the determination of the net amount of acid- or base-binding groups present. The former is spoken of as the hydrogen-ion (H^+) concentration and the latter as the titratable acidity or alkalinity.

The hydrogen-ion concentration can be determined either colorimetrically or electrometrically. The titratable acidity is determined by titration, finding the difference between the titer of the solution and whatever is accepted as the neutral point or initial acidity. Both methods serve very useful purposes in bacteriological technique. The

adjustment of media is more accurately carried out by means of the hydrogen-ion method. The titratable acidity determination is of great value in learning the buffer content of the medium, *i.e.*, its ability to resist changes in reaction on the addition of acid or alkali.

Measuring the Concentration of Hydrogen (H^+) Ions.—Pure water is neutral in reaction because it ionizes into equal numbers of hydrogen (H^+) and hydroxyl (OH^-) ions. One liter of pure water contains 0.0000001 mole of hydrogen ions. This may be written 10^{-7} mole per liter. For each H^+ ion there is a corresponding and neutralizing OH^- ion.

According to the law of mass action,

$$HOH \rightleftharpoons H^+ + OH^-$$

or

$$\frac{(H^+)(OH^-)}{HOH} = K$$

Since the concentration of the undissociated water is very great, it can be taken as a constant. The equation can be expressed as follows:

$$(H^+)(OH^-) = K$$

The numbers of H^+ and OH^- ions being equal, each must have a concentration of 1×10^{-7}. The product of the concentration of the hydrogen and hydroxyl ions is equal to 1×10^{-14}. The equation now becomes

$$(H^+) \times (OH^-) = 1 \times 10^{-14}$$

Pure water which has a hydrogen-ion concentration of 1×10^{-7} is neutral in reaction. If the hydrogen-ion concentration of a solution is smaller than 1×10^{-7}, it will have an alkaline reaction; if greater than 1×10^{-7}, it will have an acid reaction.

The term pH is defined as the logarithm of the reciprocal of the hydrogen-ion concentration. For convenience only the exponent is used in expressing pH. If a solution has a pH less than 7, it is acid in reaction; if greater than 7, it is alkaline. Most organisms grow best in culture media adjusted to a pH of about 7.0.

The relation of pH to the strength of hydrochloric acid and sodium hydroxide is given in Table 31.

Colorimetric Method.—The determination of the hydrogen-ion concentration by the colorimetric method depends upon the color changes produced in certain weakly acid or basic dyes by varying the reaction of the medium. Such dyes are called indicators. All indicators dissociate on the addition of acid or alkali. An indicator changes in color within a short distance each side of that point in the pH scale at which it is 50 per cent dissociated. At this point one-half of the dye is

undissociated and the other half is dissociated in the form of free ions. The pH at which this occurs is denoted by the symbol pK.

A short distance each side of the pK point gives a zone which is referred to as the sensitive range of the indicator. Every shade of color of the indicator in this sensitive range corresponds to a definite pH value so that by comparing the shade of the indicator with standards of known reaction the hydrogen-ion concentration of a solution can be determined. Indicators can be selected showing a certain amount of overlapping in their sensitive ranges so that the scale from pH1.0 to 10.0 can be covered.

A list of the most important pH indicators used in bacteriology and some of their characteristics are given in Table 32.

TABLE 31.—STRENGTHS OF SOLUTIONS OF HCl AND NaOH AND THEIR CORRESPONDING pH VALUES

Acid or basic	Strength of solution	Grams hydrogen per liter	Expressed in logarithms	pH
Acid	$\frac{N}{1}$ HCl	1.0	10^{-0}	0.0
Acid	$\frac{N}{10}$ HCl	0.1	10^{-1}	1 0
Acid	$\frac{N}{100}$ HCl	0 01	10^{-2}	2.0
Acid	$\frac{N}{1000}$ HCl	0.001	10^{-3}	3.0
Acid	$\frac{N}{10,000}$ HCl	0.0001	10^{-4}	4.0
Acid	$\frac{N}{100,000}$ HCl	0.00001	10^{-5}	5.0
Acid	$\frac{N}{1,000,000}$ HCl	0.000001	10^{-6}	6.0
Neutral	Pure water	0 0000001	10^{-7}	7.0
Basic	$\frac{N}{1,000,000}$ NaOH	0.00000001	10^{-8}	8.0
Basic	$\frac{N}{100,000}$ NaOH	0.000000001	10^{-9}	9.0
Basic	$\frac{N}{10,000}$ NaOH	0.0000000001	10^{-10}	10.0
Basic	$\frac{N}{1000}$ NaOH	0.00000000001	10^{-11}	11.0
Basic	$\frac{N}{100}$ NaOH	0.000000000001	10^{-12}	12.0
Basic	$\frac{N}{10}$ NaOH	0.0000000000001	10^{-13}	13.0
Basic	$\frac{N}{1}$ NaOH	0.00000000000001	10^{-14}	14.0

Buffers.—The salts of weak acids have the power of preventing pronounced changes in the reactions of solutions on the addition of

relatively large amounts of strong acids or alkalies. Substances that possess the power of resisting changes in acidity or alkalinity are spoken of as buffers.

The addition of 1 cc. of $N/10$ hydrochloric acid to 1 liter of neutral distilled water (pH7.0) gives a solution having a pH of about 4.0. The addition of 1 cc. of N/10 NaOH to 1 liter of neutral distilled water gives a solution having a pH of about 10.0. The addition of the same amount of acid or alkali to 1 liter of distilled water, in which is dissolved a few grams of sodium phosphate, produces very little, if any, change in the reaction. Sodium phosphate is classed as a buffer. This may be shown in the following reactions:

The addition of a strong acid.

$$Na_2HPO_4 + HCl \rightarrow NaH_2PO_4 + NaCl$$

The strong acid (HCl) reacts with the weak alkali (Na_2HPO_4) to give the weak acid (NaH_2PO_4) and sodium chloride. In other words, the strong HCl is replaced by the weak acid phosphate resulting in a relatively small change in the final hydrogen-ion concentration.

The addition of a strong alkali:

$$NaH_2PO_4 + NaOH \rightarrow Na_2HPO_4 + H_2O$$

TABLE 32.—COLOR CHANGES OF THE IMPORTANT BACTERIOLOGICAL INDICATORS

Indicator	Concentration recommended*	Full acid color	Full alkaline color	Sensitive range (pH)	pK†
m-Cresol purple (acid range)	0.04	Red	Yellow	1.2– 2.8	1.5
Thymol blue (acid range)	0.04	Red	Yellow	1.2– 2.8	1.5
Bromophenol blue	0.04	Yellow	Blue	3.0– 4.6	4.1
Bromochlorophenol blue	0.04	Yellow	Blue	3.0– 4.6	4 0
Bromocresol green	0.04	Yellow	Blue	3.8– 5.4	4.7
Chlorocresol green	0.04	Yellow	Blue	4.0– 5.6	4.8
Methyl red	0.02	Red	Yellow	4.4– 6.0	5.1
Chlorophenol red	0.04	Yellow	Red	4.8– 6.4	6 0
Bromophenol red	0.04	Yellow	Red	5.2– 6.8	6.2
Bromocresol purple	0.04	Yellow	Purple	5.2– 6.8	6.3
Bromothymol blue	0.04	Yellow	Blue	6.0– 7.6	7.0
Phenol red	0.02	Yellow	Red	6.8– 8.4	7.9
Cresol red	0.02	Yellow	Red	7.2– 8.8	8.3
m-Cresol purple (alkaline range)	0 04	Yellow	Purple	7.4– 9.0	8.3
Thymol blue (alkaline range)	0.04	Yellow	Blue	8.0– 9.6	8.9
Cresolphthalein	0.04	Colorless	Red	8.2– 9.8	9.4
Phenolphthalein	0 04	Colorless	Red	8 3–10.0	9.7

* Per cent of dye dissolved in 50 per cent ethyl alcohol.

† The pH at which the dye is 50 per cent dissociated.

The strong alkali (NaOH) reacts with the weak acid (NaH_2PO_4) to give the weak alkali (Na_2HPO_4) and water. The strong NaOH is replaced by the weak basic phosphate, resulting in a relatively small change in the final hydrogen-ion concentration.

The important salts commonly added to nutrient media for their buffering action include phosphates and carbonates. These compounds are particularly valuable because they are relatively nontoxic.

Bacteriological peptones contain such substances as proteoses, peptones, peptids, and amino acids, all of which are buffers. These possess both acidic and basic properties, *i.e.*, they have the power of uniting with both bases and acids. Therefore, all culture media containing peptone are well buffered, the degree of buffering being dependent upon the amount of peptone added.

Buffers are of special importance in media containing fermentable carbohydrates. In the various fermentations organic acids are produced. As the concentration of hydrogen ions increases, the pH decreases. Finally a pH is reached where the [illegible] no longer continue to multiply. Sufficient acid has accumulated to stop the activity of the organisms. This takes place usually in from 24 to 48 hr. On the other hand, if media are employed that do not contain buffering substances, the activity of the organisms will cease after a few hours. A good culture medium, besides containing the required nutrients, should be also well buffered.

For additional information on the theory and measurement of the hydrogen-ion concentration and on buffers, consult the books by Britton (1929), Clark (1928), Grant (1930), Michaelis (1926), and Van Slyke (1922).

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CHAPTER XII

ENZYMES OF BACTERIA

Catalysis may be defined as the acceleration of a reaction produced by the presence of a substance known as a catalyst. The term is compounded from the two Greek words, κατά, down, and λνειν, to loose. It means literally to destroy or to dissolve but has now come to mean the hastening of a chemical reaction.

A catalyst is an agent that accelerates a chemical reaction without itself being destroyed or changed. It may be recovered at the end of the reaction.

An enzyme or ferment may be defined as an organic catalyst elaborated by a living cell and capable of functioning independently of the presence of the cell.

An enzyme acts by catalysis, *i.e.*, it increases the velocity of a chemical reaction without itself being permanently changed. The enzyme may be recovered in the active form after the completion of the reaction. There is a close analogy between inorganic catalysts and enzymes. Hydrogen peroxide, for example, slowly decomposes into water and oxygen according to the equation:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

In the presence of an inorganic catalyst such as platinum, or the enzyme catalase, the decomposition of the peroxide is greatly accelerated and ceases only when the destruction of the compound is complete.

It is usually stated that a catalyst does not initiate a new reaction but merely speeds up one already in progress. This statement is probably true in the great majority of cases but there are some exceptions. Newer work indicates that a catalyst may initiate a new reaction and not just cause an acceleration of one already in progress.

CLASSIFICATION OF ENZYMES

The great majority of enzymes have not been purified by crystallization but by precipitation and absorption methods. Since these procedures are also used for the purification of proteins, it is never known when enzymes are free from such compounds. For this reason enzymes are classified on the basis of what they do rather than on what they are. Thus there are various classes of enzymes, such as amylolytic (starch-

hydrolyzing), proteolytic (protein-hydrolyzing), lipolytic (fat-hydrolyzing), oxidizing, and reducing. With the exception of a few of the earlier enzymes such as pepsin, trypsin, and rennin, their names end in -ase. For instance, an enzyme that acts on lactose is called lactase; one that acts on uric acid is called uricase; one that acts on the amino acid arginine is called arginase; etc.

The classification of enzymes is a difficult task, owing to the fact that it is never known when a certain preparation under examination contains only a single enzyme. Many preparations which were at one time believed to be composed of a single enzyme have been shown since to contain more than one enzyme. Evidence accumulated during the last few years seems to point to the fact that enzymes now placed into different groups are really the same enzyme acting under slightly different conditions.

One of the properties of proteins is their large molecular weights. This means that they possess the characteristics of colloids. Enzymes also give the reactions of proteins. The water content of bacteria ranges from 73 to 98 per cent. If we assume that an average bacterial cell is a cube 2μ on each side and the diameter of a colloid particle of the order of $100\mu\mu$ (0.1μ), it is difficult to picture how a cell as small as that of a bacterium can accommodate upwards of 100 different enzymes. The work on bacterial dehydrogenases indicates that it is better to assume the presence of an active group located at or near the cell surface, together with some other factor such as specific absorption, rather than the presence of many different enzymes.

Regardless of whether vital reactions are produced by one or many enzymes, a classification is believed to be essential for a clearer understanding of the subject. Table 33 is not complete but it shows the method followed in naming enzymes. The chemical reactions are included as far as it is possible to do so.

CHEMICAL NATURE OF ENZYMES

Willstätter (1922) advanced his so-called "Trager" or carrier theory to explain the chemical nature of enzymes, which appears to be accepted by a majority of the European workers. He believes that enzymes are prosthetic groups attached to colloidal proteins, carbohydrates, etc., as carriers just as hematin is a prosthetic group attached to a colloidal protein carrier known as globin to give hemoglobin. The enzyme possesses a special reactive group which has a specific affinity for definite groups of the substrate. This explains the specificity of enzyme actions. The enzyme may unite with the substrate but is not active unless attached to a colloidal carrier. The colloidal carrier is exchangeable for another carrier. A destruction of the colloidal properties of the aggregate

TABLE 33.—CLASSIFICATION OF ENZYMES

I. Hydrolyzing enzymes

A. Esterases

The enzymes catalyze the reaction

$$R{\cdot}COO{\cdot}R' + H_2O \rightarrow R{\cdot}COOH + R'OH$$

The acid may be a higher or a lower fatty acid and R'OH may be glycerol or a simple aliphatic or aromatic alcohol or a carbohydrate

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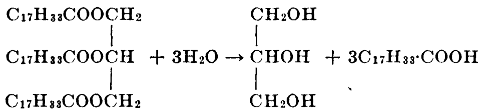
1. Lipases

a. Glyceridases + glycerides → glycerol + higher fatty acids



Triolein Glycerol Oleic acid

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2. Butyrases + lower esters → alcohols + lower fatty acids

$$CH_3{\cdot}COO{\cdot}C_2H_5 + H_2O \rightleftharpoons C_2H_5OH + CH_3{\cdot}COOH$$

Ethyl acetate Alcohol Acetic acid

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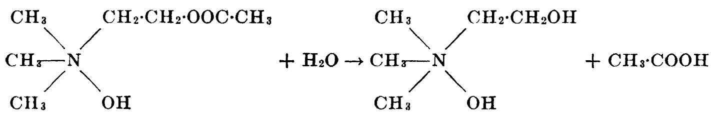
3. Pectase + pectin → pectic acid + methyl alcohol (CH_3OH)

---

4. Cholinesterase + choline esters → choline + acetic acid



Acetylcholine Choline Acetic acid

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5. Chlorophyllase + chlorophyll-*a* → chlorophyllide-*a* + phytol ($C_{20}H_{39}OH$)

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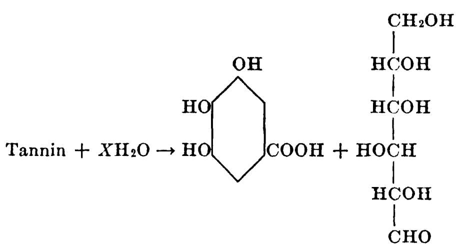
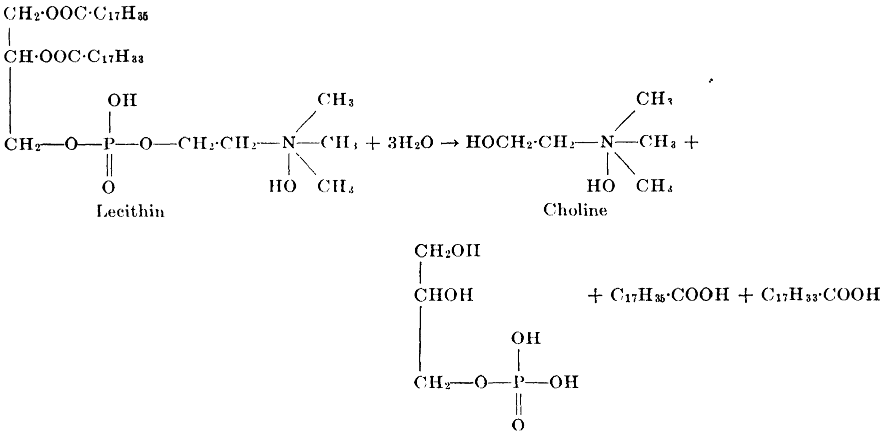
6. Tannase + tannin → gallic acid + glucose



Gallic acid Glucose

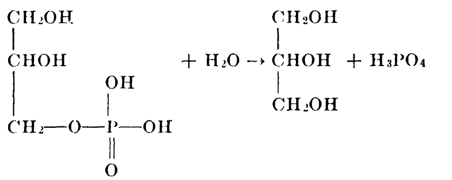
TABLE 33.—(*Continued*)

7. Phosphatases
   a. Lecithinase + lecithin → glycerophosphoric acid + choline + fatty acids



Lecithin, Choline, Glycerophosphoric acid, Stearic acid, Oleic acid

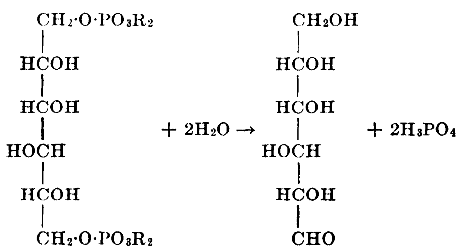
b. Phosphomono-esterase + monoesters of *o*-phosphoric acid → alcohol + phosphoric acid



Glycerophosphoric acid, Glycerol, Phosphoric acid

c. Phosphodiesterase + diesters of phosphoric acid → alcohol + monoester

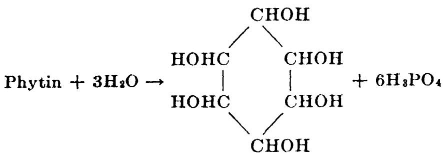
d. Hexosediphosphatase + hexosediphosphoric acid → hexose + phosphoric acid



Hexosediphosphate, Glucose, Phosphoric acid

TABLE 33.—(*Continued*)

e. Phytase + phytin → inositol + phosphoric acid

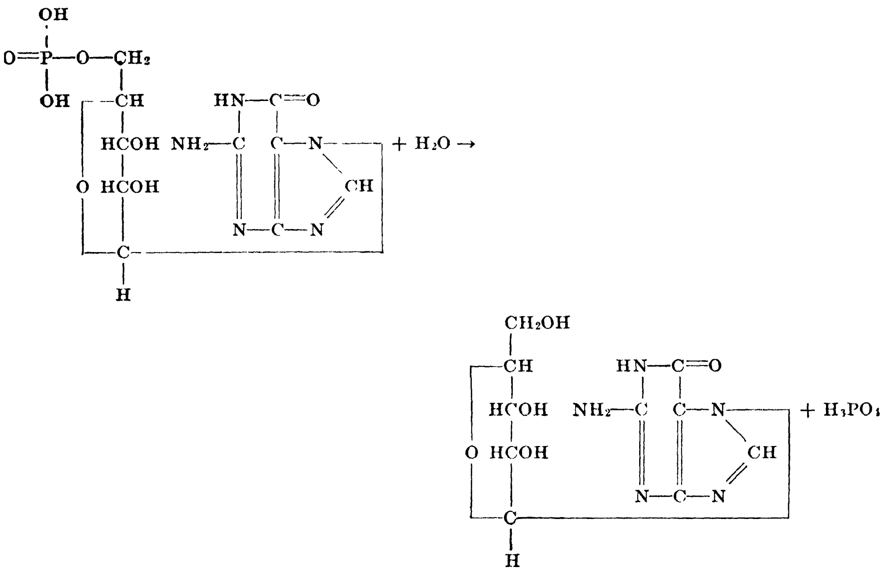


Inositol Phosphoric acid

f. Polynucleotidase + nucleic acid → mononucleotides

g. Phosphonuclease + nucleic acid → mononucleosides + phosphoric acid

h. Nucleotidase + nucleotide → nucleoside + phosphoric acid



Guanylic acid Guanine riboside Phosphoric acid

8. Cholesterase + cholesterol esters → cholesterol + R·COOH

9. Sulfatase + sulfuric acid esters of phenols → phenols + $KHSO_4$

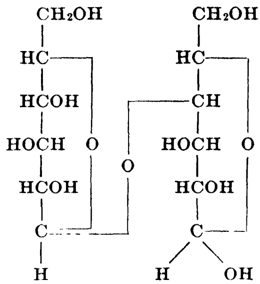
TABLE 33.—(*Continued*)

B. Carbohydrases.

The enzymes catalyze the hydrolysis of carbohydrates to compounds that are simpler and generally more soluble

1. Amylase + starch → α-maltose

$(C_6H_{10}O_5)x + XH_2O \rightarrow$

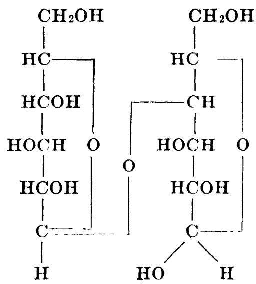


Soluble starch

Maltose (glucose-4-α-glucoside)

2. Cellulase + cellulose → cellobiose

$(C_6H_{10}O_5)x + XH_2O \rightarrow$



Cellulose

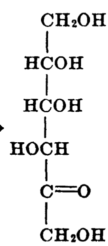
Cellobiose (glucose-4-β-glucoside)

3. Cytase + hemicelluloses → simple sugars having formula $C_6H_{12}O_6$

4. Lichenase + lichenin → cellobiose (see cellulase for formula)

5. Inulase + inulin → fructose (levulose).

$(C_6H_{10}O_5)x + XH_2O \rightarrow$

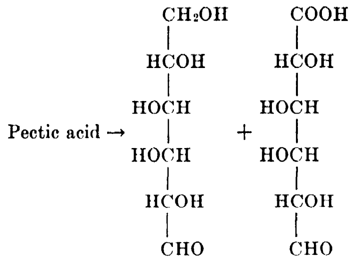


Inulin

Fructose

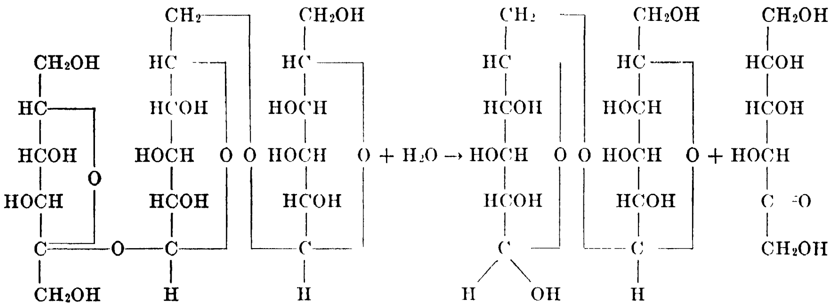
TABLE 33.—(*Continued*)

6. Pectinase + pectic acid → galactose + galacturonic acid.



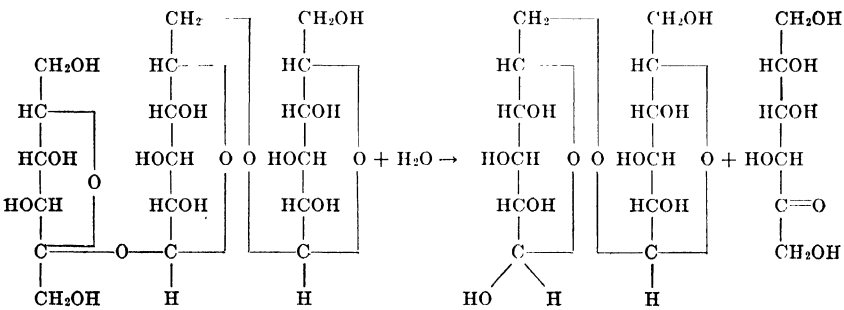
Galactose Galacturonic acid

7. Raffinase + raffinose → melibiose + fructose



Raffinose Melibiose (glucose-6-α-galactoside) Fructose

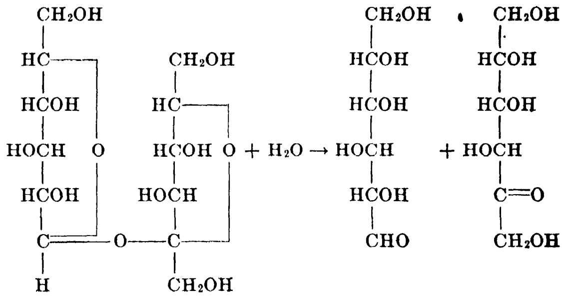
8. Gentianase + gentianose → gentiobiose + fructose



Gentianose Gentiobiose (glucose-6-β-glucoside) Fructose

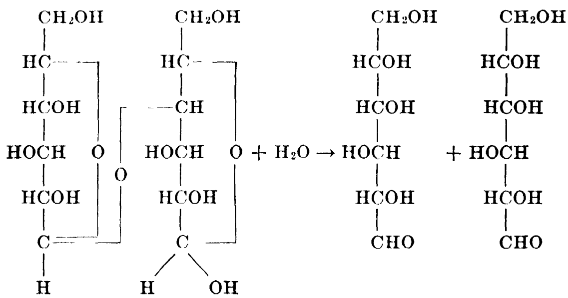
TABLE 33.—(*Continued*)

9. Invertase + sucrose → glucose + fructose



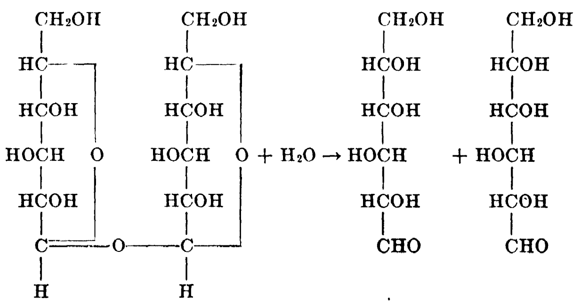
Sucrose Glucose Fructose

10. Maltase + maltose → glucose + glucose



Maltose (glucose-4-α-glucoside) Glucose Glucose

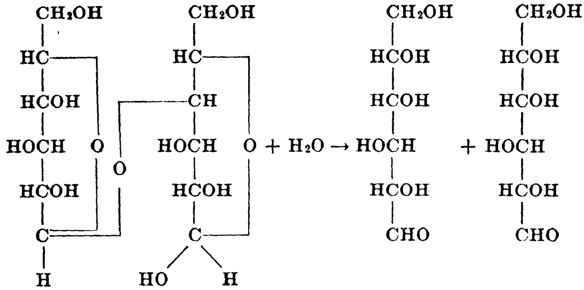
11. Trehalase + trehalose → glucose + glucose



Trehalose (glucose-1-α-glucoside) Glucose Glucose

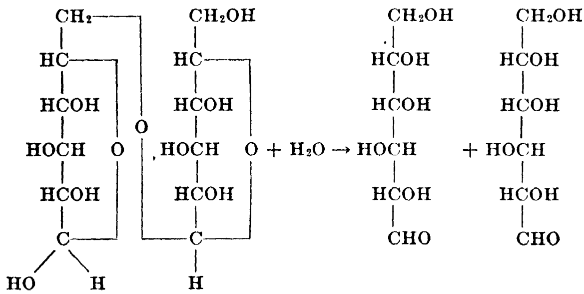
TABLE 33.—(*Continued*)

12. Cellobiase + cellobiose → glucose + glucose



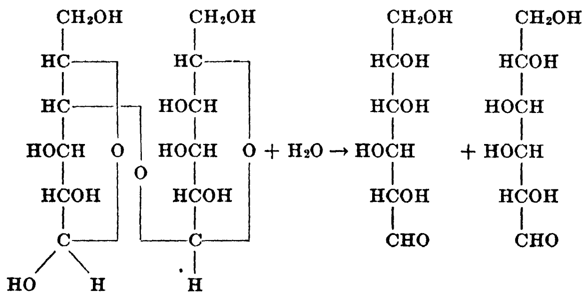
Cellobiose (glucose-4-β-glucoside) — Glucose — Glucose

13. Gentiobiase + gentiobiose → glucose + glucose



Gentiobiose (glucose-6-β-glucoside) — Glucose — Glucose

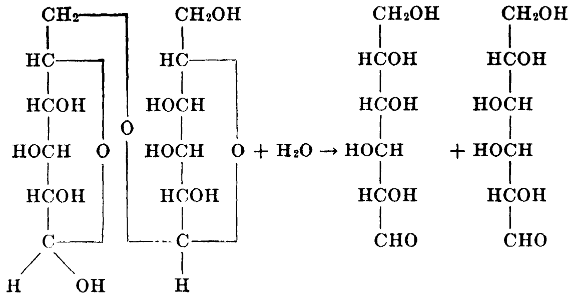
14. Lactase + lactose → glucose + galactose



Lactose (glucose-4-β-galactoside) — Glucose — Galactose

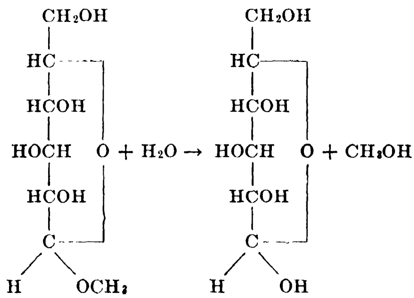
TABLE 33.—(*Continued*)

15. Melibiase + melibiose → glucose + galactose



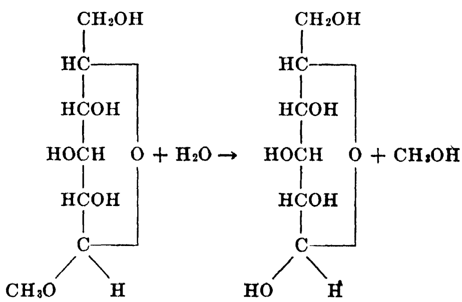
Melibiose (glucose-6-α-galactoside) Glucose Galactose

16. α-Glucosidase + α-glucosides → α-glucose + alcohol or phenol residue



α-methyl glucoside α-glucose Methyl alcohol

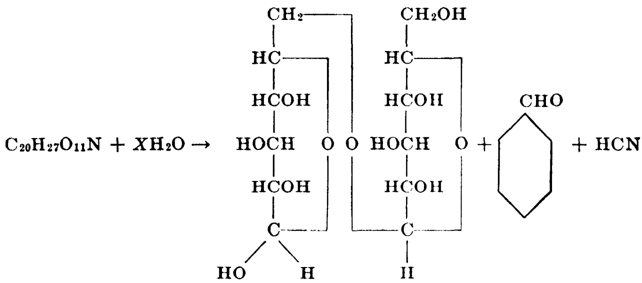
17. β-Glucosidase + β-glucosides → β-glucose + alcohol or phenol residue



β-methyl glucoside β-glucose Methyl alcohol

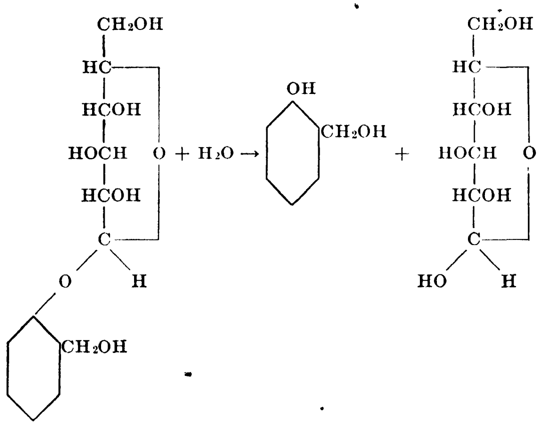
TABLE 33.—(*Continued*)

18. Amygdalase + amygdalin → gentiobiose + benzaldehyde + hydrocyanic acid



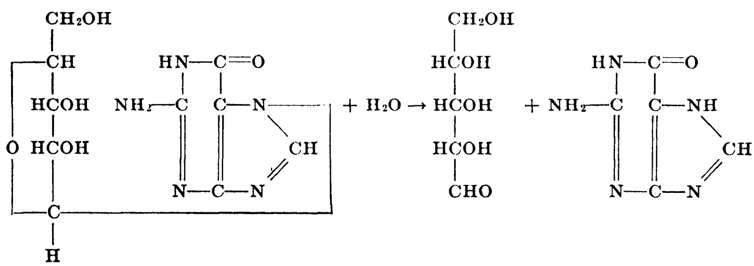
Amygdalin — Gentiobiose (glucose-6-β-glucoside) — Benzaldehyde — Hydrocyanic acid

19. Emulsin + salicin → saligenin + β-glucose



Salicin — Saligenin (*O*-hydroxy benzyl alcohol) — β-glucose

20. Nucleosidase + nucleoside → sugar + purine bases



Guanine riboside — Ribose — Guanine

C. Enzymes hydrolyzing nitrogen compounds
Compounds having smaller molecular weights are produced

TABLE 33.—(*Continued*)

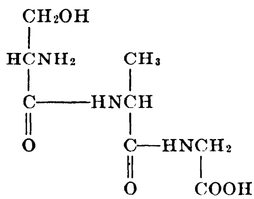
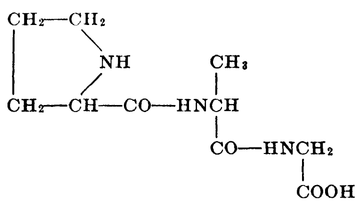
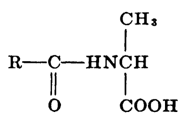
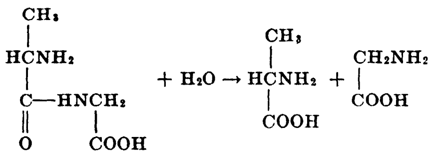
1. Proteinases *a.* Rennin + casein → paracasein	
b. Pepsin + native proteins → proteoses + peptones	
c. Trypsin + native proteins, proteoses, peptones, peptids → polypeptids and amino acids	
d. Erepsin + proteoses, peptones, polypeptids → amino acids	
e. Papain + native proteins → polypeptids and dipeptids	
f. Bromelin + native proteins → polypeptids and dipeptids	
g. Keratinase + keratins → amino acids	
h. Protaminases + protamines with terminal arginine group → arginine + residue	
2. Peptidases *a.* Aminopolypeptidases	Attack amino acid on the amino end of a polypeptid chain  Seryl-alanyl-glycine The amino acid serine is liberated on hydrolysis
b. Prolinase	Attacks proline on the imino end of peptid chain  Prolyl-alanyl-glycine
c. Carboxypolypeptidase	Attacks polypeptids having free COOH groups  The amino acid alanine is liberated on hydrolysis

TABLE 33.—(*Continued*)

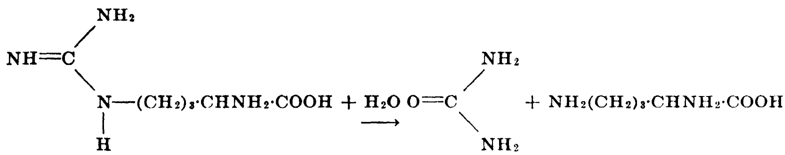
d. Dipeptidases + dipeptids → amino acids



Alanyl-glycine Alanine Glycine

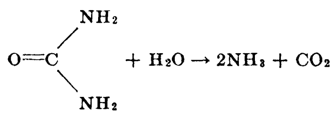
3. Desamidases

a. Arginase + arginine → urea + ornithine



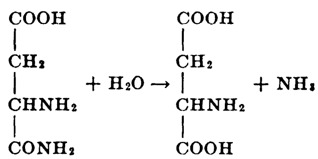
Arginine Urea Ornithine

b. Urease + urea → ammonia + carbon dioxide



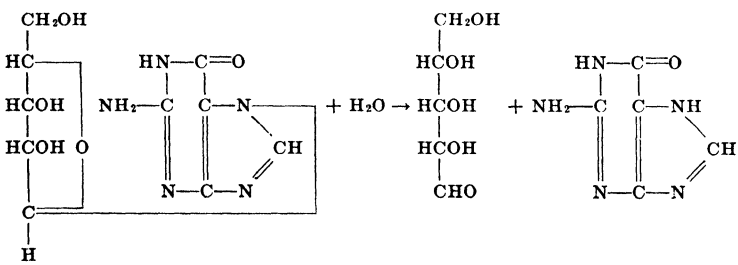
Urea Ammonia

c. Asparaginase + asparagine → aspartic acid + ammonia



Asparagine Aspartic acid

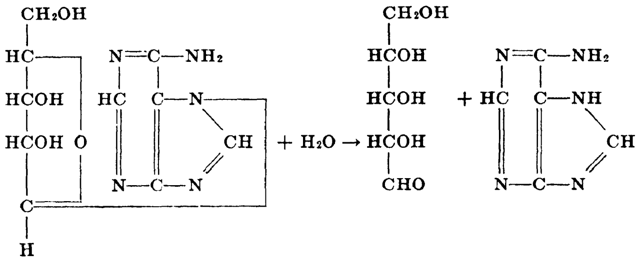
d. Guanosine desamidase + guanosine → guanine + pentose



Guanosine Ribose Guanine

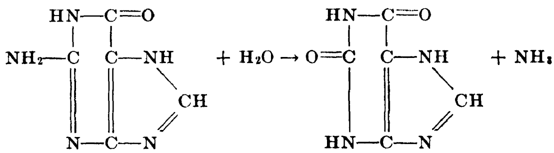
TABLE 33.—(*Continued*)

e. Adenosine desamidase + adenosine → adenine + pentose



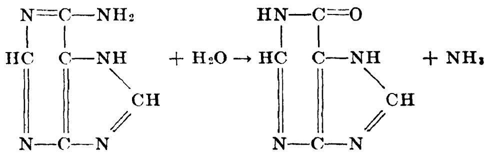
Adenosine Ribose Adenine

f. Guanase + guanine → xanthine + ammonia



Guanine Xanthine

g. Adenase + adenine → hypoxanthine + ammonia



Adenine Hypoxanthine

II. Desmolyzing enzymes

Enzymes that do not catalyze hydrolytic reactions are grouped under the desmolases. This group comprises those enzymes involved in the processes of respiration and metabolism. Normally such enzymes are not secreted into the medium but remain inside the cell

A. Zymase complex. This enzyme represents a complex of several enzymes and coenzymes	+	*d*-glucose, *d*-fructose, *d*-mannose, *d*-galactose, glyceric aldehyde, and hydroxyacetone	→ alcohol + CO_2

B. Enzymes involved in oxidation reduction

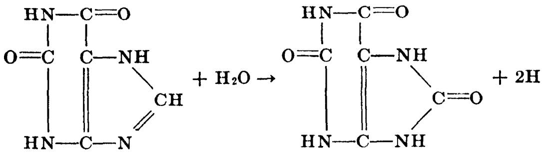
1. The dehydrogenases

These enzymes are responsible for the activation of the molecules of the metabolites so that they can be oxidized in the presence of oxygen or a suitable reducible substance. They convert nonreducing substances into reducing substances. The aerobic dehydrogenases require neither coenzyme nor cytochrome systems whereas the anaerobic dehydrogenases catalyze reactions between metabolites and carriers

TABLE 33.—(*Continued*)

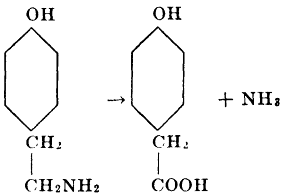
Aerobic dehydrogenases

a. Xanthine dehydrogenase + xanthine and hypoxanthine → uric acid



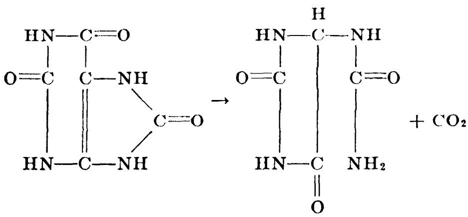
Xanthine — Uric acid

b. Tyramine dehydrogenase + tyramine → *p*-oxyphenyl acetic acid



Tyramine — *p*-oxyphenyl acetic acid

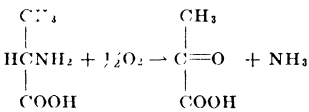
c. Uricase + uric acid → allantoin + CO_2



Uric acid — Allantoin

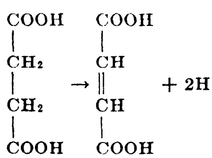
Anaerobic dehydrogenases

a. *d*-Amino acid dehydrogenase + amino acids → corresponding keto acids



Alanine — Pyruvic acid

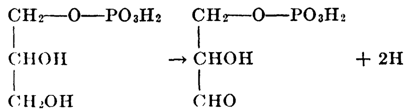
b. Succinic dehydrogenase + succinic acid → fumaric acid



Succinic acid — Fumaric acid

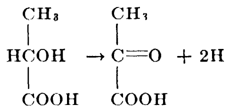
TABLE 33.—(*Continued*)

c. Glycerophosphoric dehydrogenase + glycerophosphoric acid → glyceric aldehyde phosphoric acid

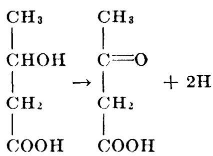


Glycerophosphoric acid   Glyceric aldehyde phosphoric acid

d. Lactic dehydrogenase + lactic acid → pyruvic acid

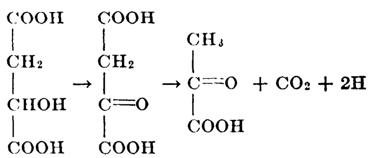


e. β-Hydroxybutyric dehydrogenase + β-hydroxybutyric acid → acetoacetic acid



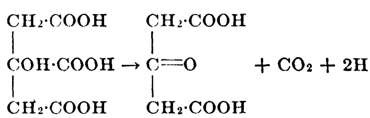
β-Hydroxybutyric acid   Acetoacetic acid

f. Malic dehydrogenase + malic acid → pyruvic acid



Malic acid   Oxalacetic acid   Pyruvic acid

g. Citric dehydrogenase + citric acid → acetone dicarboxylic acid



Citric acid   Acetone dicarboxylic acid

h. Alcohol dehydrogenase + alcohols → aldehydes

$$R{\cdot}CH_2OH \rightarrow R{\cdot}CHO + 2H$$

Alcohol   Aldehyde

TABLE 33.—(*Continued*)

i. Glucose dehydrogenase + *d*-glucose → *d*-gluconic acid

$$\begin{array}{l} CH_2OH \\ | \\ (CHOH)_4 \\ | \\ CHO \end{array} + H_2O \rightarrow \begin{array}{l} CH_2OH \\ | \\ (CHOH)_4 \\ | \\ COOH \end{array} + 2H$$

d-Glucose   *d*-Gluconic acid

j. Aldehyde mutase → aldehydes → saturated fatty acid + alcohol (dehydrogenase)

(1) $CH_3{\cdot}CHO + H_2O \rightarrow CH_3{\cdot}COOH + 2H$
Aldehyde   Acetic acid

(2) $2CH_3{\cdot}CHO + H_2O \rightarrow CH_3{\cdot}COOH + CH_3{\cdot}CH_2OH$
Aldehyde   Acetic acid   Alcohol

k. Glyceric aldehyde phosphoric acid dehydrogenase → glyceric aldehyde phosphoric acid → phosphoglyceric acid

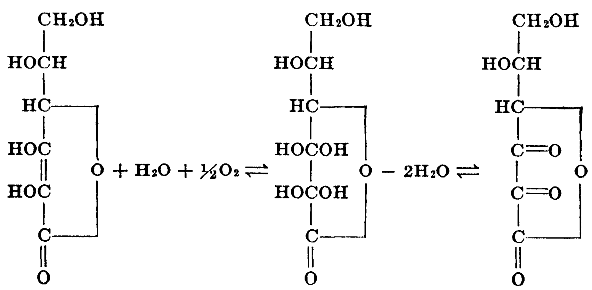
$$\begin{array}{l} CH_2{-}O{-}PO_3H_2 \\ | \\ CHOH \\ | \\ CHO \end{array} + H_2O \rightarrow \begin{array}{l} CH_2{-}O{-}PO_3H_2 \\ | \\ CHOH \\ | \\ COOH \end{array}$$

Glyceric aldehyde phosphoric acid   Phosphoglyceric acid

2. The oxidases
The oxidases appear to function by activating oxygen so that it will quickly oxidize slowly autooxidizable compounds. They are metalloproteins being inhibited by hydrogen sulfide and potassium cyanide. They do not form hydrogen peroxide as a result of the reduction of oxygen. Oxidases are active only under aerobic conditions

a. Cytochrome oxidase + reduced cytochrome → oxidized cytochrome (indophenol oxidase)

b. Ascorbic acid oxidase + reduced ascorbic acid → oxidized ascorbic acid



Reduced ascorbic acid   Dehydroascorbic acid (oxidized)

TABLE 33.—(*Continued*)

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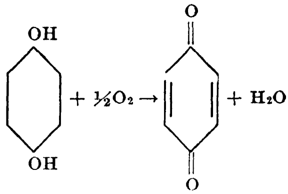
c. Polyphenol oxidase (laccase) + polyphenols → corresponding quinones



p-dihydroxy benzene — Quinone

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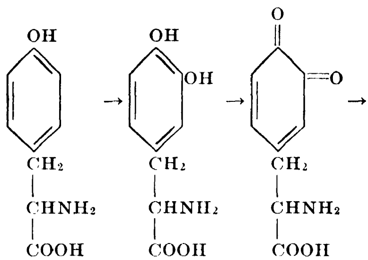
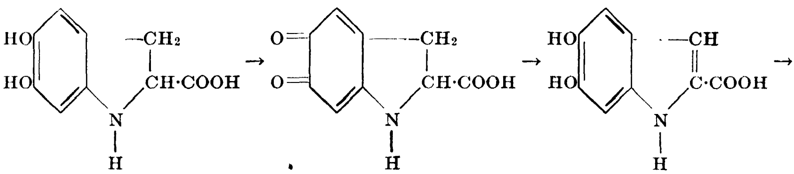
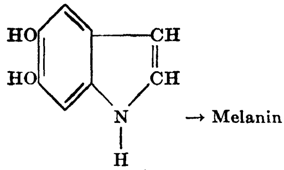
d. Tyrosinase (tyrosine oxidase) + tyrosine → melanine (black pigment)



Tyrosine — 3:4-dihydroxy phenylalanine — 3:4-quinone of phenylalanine



5:6-dihydroxy-dihydroindole-2-carboxylic acid — Hallochrome — 5:6-dihydroxy-indole carboxylic acid



5:6-dihydroxyindole

---

e. Dopa oxidase + 3:4-dihydroxy phenylalanine (dopa) → melanin (see under tyrosinase)

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f. Luciferase + luciferin → oxyluciferin + light produced by bioluminescent organisms

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TABLE 33.—(*Continued*)

3. Catalase

This enzyme is a chromoprotein, being composed of a colored prosthetic group united to a protein. The prosthetic group appears to be a hematin compound. It is found in most plant and animal cells and in aerobic bacteria. The most important function of the enzyme is to decompose hydrogen peroxide into water and molecular oxygen. There is some evidence that the oxygen released may be used in oxidation reactions

Catalase + hydrogen peroxide → water + molecular oxygen

$$\underset{\text{Hydrogen peroxide}}{2H_2O_2} \rightarrow 2H_2O + O_2$$

4. Peroxidase

Like catalase, this enzyme is also a compound of hematin with a protein. However, the proteins in the two enzymes are different. Peroxidase catalyzes the oxidation of certain substances by hydrogen peroxide. In the presence of certain oxidizable substances the decomposition of the peroxide is very rapid and is accompanied by the oxidation of the substance

Peroxidase + hydrogen peroxide → water + atomic oxygen

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{peroxidase}\cdot H_2O_2$$

$$\underset{\text{Enzyme-peroxide compound}}{\text{Peroxidase}\cdot H_2O_2} + \underset{\text{Oxidizable substance}}{RH_2} \rightarrow \text{peroxidase} + \underset{\text{Oxidized substance}}{R} + 2H_2O$$

(enzyme + colloidal carrier) results in a disappearance of enzymatic activity.

Opposing this theory is the viewpoint held by the American workers. They believe that enzymes are specific and definite chemical compounds, probably of a protein nature, and that the arrangement of the different groups in the protein molecule is responsible for both enzyme activity and enzyme specificity. In support of this theory is the preparation of a considerable number of enzymes in crystalline form all of which give positive tests for proteins.

The enzymes that have been crystallized include urease, pepsin, trypsin, chymotrypsin, chymotrypsinogen, trypsinogen, a lipase, the yellow oxidation enzyme of Warburg and Christian, carboxypolypeptidase, pepsinogen, papain, etc.

In conclusion it may be stated that available evidence appears to support both theories. Some enzymes appear to be true prosthetic groups, which can be separated from the specific protein carrier by physical methods. Other enzymes, such as the crystalline catalysts, do not appear to be prosthetic groups united to protein carriers. Their enzymatic activities are believed to be due to a particular arrangement of the amino acids in the enzyme molecules.

FACTORS THAT INFLUENCE ENZYME ACTIONS

Enzyme actions are greatly influenced by such factors as temperature, hydrogen-ion concentration, ultraviolet light, activators and inhibitors, concentration of enzyme and substrate, etc. The same factors that influence the growth of microorganisms also affect the action of enzymes. As a general rule, enzymes are more resistant to unfavorable environmental conditions than the cells producing them. For example, it is known that if dried yeast is heated at 100°C. for 6 hr. it loses its power of growth and multiplication but still retains the power of fermenting sugar to alcohol and carbon dioxide.

Effect of Temperature.—The velocity of the action of an enzyme on its substrate is accelerated by an increase in temperature. This continues until a maximum is reached, after which the action gradually decreases until the enzyme is finally destroyed. The velocity of enzyme actions is approximately doubled for each 10° increase in temperature.

Each enzyme has its own characteristic optimum temperature. That temperature above which an enzyme no longer shows any activity is known as the maximum temperature. That temperature below which an enzyme cannot function is known as the minimum temperature. The optimum, maximum, and minimum temperatures are influenced by several factors such as concentration of enzyme, nature and concentration of substrate upon which the enzyme acts, the hydrogen-ion concentration of the medium, and the presence of activating and paralyzing substances.

Most enzymes in solution are more or less stable at temperatures below 45°C., but above 50°C. inactivation rapidly increases with a rise in temperature. The majority of enzymes are destroyed at temperatures of 70 to 80°C. On the other hand, enzymes in the dried state are more resistant to high temperatures than the same ones in solution. It has been shown that dried rennin is only slowly destroyed at 158°C.

Effect of pH.—As is true for bacteria, enzymes are also greatly influenced by the hydrogen-ion concentration of the solution. Some enzymes act best in a neutral solution; others prefer alkaline solutions; still others do not function unless the solutions are acid in reaction. There exists for every enzyme a maximum, an optimum, and a minimum hydrogen-ion concentration under certain specified conditions. The optimum pH of an enzyme varies with the substrate, the source of the enzyme, and the buffer used.

Effect of Ultraviolet Light.—Ultraviolet light either destroys or modifies the action of enzymes. A destructive action usually occurs only in the presence of oxygen although some exceptions have been reported.

The rate of destruction is practically independent of temperature but is affected by pH and other environmental conditions.

In most cases the injurious effect of ultraviolet light is greatest at the optimum hydrogen-ion concentration. For example, Seligsohn (1926) found that unirradiated blood catalase was more active at pH6.8 than at 6.2 or 7.3. After ultraviolet treatment, the reverse was true.

Purified enzymes are more easily destroyed by light than the same ones in less purified preparations. Impurities of the substrates, especially proteins, may afford considerable protection. In general the greater the degree of purification of an enzyme the more susceptible it becomes to the toxic light rays.

The addition of small amounts of fluorescent substances, such as eosin, to an enzyme solution increases the toxic effect of the light rays. This same phenomenon applies also to a bacterial suspension (see page 198).

Activators and Inhibitors.—Some substances increase the activity of enzymes and others produce the reverse effect. The former are spoken of as activators; the latter are known as inhibitors. These substances may be either specific or nonspecific. The specific activators may be more appropriately referred to as coenzymes and will be discussed on page 279.

Pepsins.—These are active only in acid solutions. The inactive form is known as pepsinogen or propepsin and becomes activated on the addition of hydrogen ions. The greatest activity occurs at a pH of about 1.6.

An active pepsin may be inhibited by the addition of OH ions (alkalies). If the solution is adjusted to a pH higher than 6.8, the enzyme is destroyed.

Papainases.—The two best known representatives are cathepsin and papain. The former is found in animal cells; the latter is present in the cells of many plants, especially in papaya, the fruit of the melon tree. The optimum pH range of these proteinases is from 4 to 7.

A sulfydryl group is believed to be an essential part of the active enzymes. Oxidation renders the enzymes "inactive" and reduction restores their activity. Activation can be effected by hydrogen sulfide, cysteine, glutathione, or HCN.

This may be represented as follows:

$$\underset{\text{Inactive papain}}{PSSP} + \underset{\text{Reducing agent}}{2GSH} \rightarrow \underset{\text{Active papain}}{2PSH} + GSSG$$

Tryptases.—The tryptases or trypsins have an optimum pH range of 8 to 9. Inactive trypsin or trypsinogen is usually activated by a substance known as enterokinase. Northrop (1932*a*,*b*) and Kleiner and

Tauber (1934) reported the isolation of trypsin from pancreatic tissue, which does not require enterokinase for activity. More recently Kunitz and Northrop (1933) isolated a crystalline trypsin from beef pancreas, which was inactive both in the absence and presence of enterokinase.

Catalase.—This enzyme decomposes hydrogen peroxide into water and oxygen and at the same time furnishes oxygen for dehydrogenation. Catalase is inhibited in its action by ferrous sulfate, sodium cyanide, and phosphates but not affected by carbon monoxide, carbon dioxide, cysteine, and glutathione.

Urease.—The enzyme hydrolyzes urea to ammonia and carbon dioxide. Its optimum pH is about 7.0.

Jacoby (1933) reported that the enzyme is inactivated by cupric chloride, mercuric chloride, and silver nitrate. Sumner, Lloyd, and Poland (1933) believed that the destructive action of the metal salts was due to a sulfydryl group (SH) in the urease molecule. Urease inactivated by the heavy metals may be reactivated by the addition of hydrogen sulfide. The same holds true for benzyl mercuric chloride. Quastel (1932) found basic dyes to inhibit the action of urease but the acid dyes were without effect. Urea, amino acids, methylamine, and hydroxylamine protected the enzyme against such an inhibiting action of the basic dyes.

Inactivation of Enzymes by Proteases.—In general, proteolytic enzymes produce an inhibitory or destructive action on other enzymes. This would indicate that those enzymes which are destroyed are proteins or are associated with colloidal carriers that are proteins. Tauber (1930) found that urease, stabilized by gum acacia, was completely destroyed by trypsin. Sumner, Kirk, and Howell (1932) reported that urease was also digested by pepsin and papain-H_2S. Tauber and Kleiner (1933) found maltase to be readily destroyed by trypsin. The same workers (1932) stated that rennin was rapidly digested by both pepsin and trypsin. Tauber and Kleiner (1934) and Northrop and Kunitz (1932) found that trypsin was digested by pepsin at pH2.0, but at pH5.5 pepsin was digested by trypsin.

These are a few of the many observations that have been reported. In general, it may be concluded that those enzymes which are digested by proteases are probably proteins.

For further reading see Tauber (1935) and Northrop (1937).

Effect of Concentration of Substrate and Enzyme.—The velocity of an enzymatic reaction increases or decreases with an increase in the concentration of substrate. If the substrate concentration is small in proportion to the amount of enzyme, an increase in the substrate content may increase the velocity of the reaction. Sucrose is hydrolyzed to glucose and levulose by the enzyme sucrase. The velocity of hydrolysis

is increased by increasing the sucrose concentration up to 4 or 5 per cent. Above this concentration the relative amount of sugar hydrolyzed becomes progressively less as the sugar concentration is gradually increased. It may be concluded that a strong solution of cane sugar diluted to a concentration of about 5 per cent will be more efficiently hydrolyzed by sucrase than a more concentrated solution.

The time required for sucrase to hydrolyze cane sugar is proportional to the concentration of the enzyme. In general this is true for all enzymes when the amount of enzyme is much smaller than the concentration of substrate so that all of the enzyme can combine with the latter. When large amounts of enzyme are used, however, the velocity appears to be proportional to the square root of the concentration of the enzyme.

SPECIFICITY OF ENZYMES

Inorganic catalysts, such as nickel, platinum, and palladium, are able to catalyze many reactions. On the other hand, enzymes show a greater degree of specificity with respect to the substrates acted upon. Carbohydrases do not act on proteins; lipases are without effect on carbohydrates; proteases do not attack fats; etc. Frequently, marked differences exist in their specificity. In general, an enzyme that ferments one disaccharide will not attack another. A number of enzymes are known to attack proteins and some degree of specificity is shown. Lipases do not show a high degree of specificity, the same enzyme being capable of attacking several kinds of fats. It is generally believed that enzymes can act only upon substances having a stereoconfiguration compatible with their own asymmetry. The enzyme bears the same relationship to its specific substrate as a key does to a lock.

ADAPTIVE AND CONSTITUTIVE ENZYMES

Usually an organism is capable of secreting all its enzymes irrespective of the composition of the medium in which it is grown. These are known as the constitutive enzymes. They appear to be essential enzymes carried by the cell.

In some instances an organism can be made to elaborate an enzyme, which it does not form otherwise. The continued action of an organism on a specific substrate will finally result in the utilization of the compound. These are known as adaptive enzymes. For example, arabinose is not attacked by *Salmonella enteritidis* but several transfers to the same medium finally result in the fermentation of the sugar. The enzymes appear only when the specific substrate is present and are not believed to be essential enzymes. Such action is usually a transient one, the acquired characteristic being quickly lost on transferring the organism to a medium lacking in the specific substrate.

Rahn (1938) stated that the adaptive enzymes are elaborated by most cells under the following conditions: (1) The reaction does not take place unless it is needed. (2) The mechanism necessary to produce this reaction is not preformed in the cell, but is formed only when needed. (3) The mechanism is not inherited, but the ability to set up this mechanism in case of need is inherited. (4) All reactions involved are highly specific, chemically speaking. Rahn, Hegarty, and Deuel (1938) grew *Streptococcus lactis* in glucose broth, separated the cells by centrifugation, and resuspended them in phosphate buffer (pH7.0) with the addition of glucose or galactose. The suspensions were so concentrated that growth failed to take place. A parallel set of suspensions was prepared with cells grown in galactose broth. The results are shown in Table 34. The organisms grown in glucose broth for 12 hr. cannot ferment galactose but those grown in galactose broth ferment both sugars. Glucose is always fermented even when the bacteria are grown without any sugar. This indicates that the enzymes are constitutive. On the other hand the enzyme that attacks galactose is adaptive.

TABLE 34.—MILLIGRAMS OF LACTIC ACID PRODUCED BY *Streptococcus lactis* GROWN UNDER DIFFERENT CONDITIONS

Time, hr.	Cells grown in glucose broth, then suspended in		Cells grown in galactose broth then suspended in	
	Glucose	Galactose	Glucose	Galactose
1	240	0	160	180
2	360	0	300	270
3	420	0	370	400
4	470	0	420	410

The most important factor in adaptation appears to be the age of the cells when they are placed in contact with the new substrate. The older the cells are, the slower will be the adaptation. The delay is necessary for the cells to build the mechanism that is responsible for the elaboration of the enzyme acting on galactose.

Karström (1938) grew the organism *Leuconostoc mesenteroides* in a medium containing only one of a series of sugars. After growth the organisms were separated from the medium, washed, and then inoculated into a series of tubes, each containing one of the carbohydrates. The results are given in Table 35. It may be seen that glucose, levulose, and mannose are fermented, indicating that the enzymes for these sugars are constitutive. On the other hand, the enzymes that ferment galactose, arabinose, and lactose are developed only in the presence of the appropriate substrate, indicating that the enzymes for these sugars are

adaptive. Maltose is an exception in that the enzyme appears in the absence of any sugar.

TABLE 35.—FORMATION OF CONSTITUTIVE AND ADAPTIVE ENZYMES

Carbohydrate	Fermentation reaction				
	Glucose, levulose, mannose	Galactose	Arabinose	Maltose	Lactose
Glucose	+	–	–	–	–
Galactose	+	+	–	–	–
Arabinose	+	–	+	–	–
Sucrose	+	–	–	–	–
Maltose	+	–	–	+	–
Lactose	+	+	–	–	+
No sugar	+	–	–	+	–

The constitutive enzymes may be subdivided into two groups. The enzymes in one group always appear in approximately the same amount regardless of the composition of the medium. The enzymes in the other group vary in the amounts produced depending upon the composition of the medium. The presence of certain nutrients causes an increased elaboration of the specific enzymes. For example, an organism is capable of hydrolyzing starch to maltose. In the absence of starch only a minute amount of the specific enzyme is secreted. If starch is added to the medium, an increased amount of the enzyme is elaborated by the cells.

The production of adaptive enzymes is not always the result of a specific response to the presence of the homologous substrate in the culture medium. For example, calcium bears a definite relation to the formation of gelatinase by some bacterial species. In other words, the production of an enzyme may be stimulated by the presence of substances in the medium unrelated to the substrate attacked by the enzyme.

For excellent reviews of the subject see the contributions by Rahn (1938), Karström (1938), and Dubos (1940).

EXTRACELLULAR ENZYMES

Enzymes of bacteria may be divided into two groups depending upon whether they are secreted into the surrounding culture medium or remain confined within the cell. The enzymes that belong to the first group are known as the extracellular or exoenzymes; those which remain within the bacterial cell are known as the intracellular or endoenzymes.

It has been recognized from the time of Pasteur that the changes produced by organisms acting on carbohydrates (fermentation) and on

proteins (protein decomposition and putrefaction) are brought about by the enzymes that they elaborate. Enzymes are capable of producing their specific actions in the complete absence of the living cell. This may be easily shown in the case of the extracellular enzymes by centrifugating a culture of organisms, passing the clear supernatant liquid through a suitable filter, and demonstrating enzyme action by adding the filtrate to the appropriate substrate.

Extracellular enzymes may be demonstrated also by incorporating insoluble, indiffusible compounds into solid culture media. The presence of caseolytic, hemolytic, amylolytic (diastatic), and lipolytic enzymes may be shown by employing appropriate substrates.

Hydrolysis of Starch.—Starch is found widely distributed throughout the plant kingdom. It occurs in the form of granules in grains, fruits, and tubers.

Starch grains differ in size and shape according to the source. Also, starch grains from different sources differ somewhat in composition. The chief constituents of starch grains are known as β-amylose and α-amylose. The β-amylose is soluble in water and is colored blue by iodine solution. The α-amylose is insoluble in water and gives no color with iodine solution.

Soluble starch is produced by treating the granules with dilute hydrochloric acid. It is then soluble in warm water with the formation of a clear, limpid solution. The hydrochloric acid acts on the α-amylose, causing it to disintegrate easily when heated with water.

Many organisms are capable of elaborating the enzyme amylase, which is capable of hydrolyzing starch to maltose. Amylase is an extracellular enzyme secreted by organisms to convert the indiffusible compound into diffusible maltose. The maltose is then capable of entering the cell where it is utilized. Maltose is probably further hydrolyzed to glucose intracellularly by means of the enzyme maltase. The only function of amylase is to hydrolyze starch to maltose. The products of fermentation of starch are the result of the intracellular utilization of glucose.

The presence of amylase may be demonstrated by filtering a culture and mixing some of the filtrate with starch. The disappearance of the starch indicates the presence of amylase. This may be detected by the addition of a few drops of a dilute iodine solution. A blue color indicates the presence of starch; a brown color indicates the complete hydrolysis of the starch to maltose. A simple procedure is to streak a loopful of a culture over the surface of a starch agar plate. After incubation the plate is flooded with a dilute solution of iodine. The absence of a blue color at some distance from the bacterial growth indicates the extracellular hydrolysis of starch.

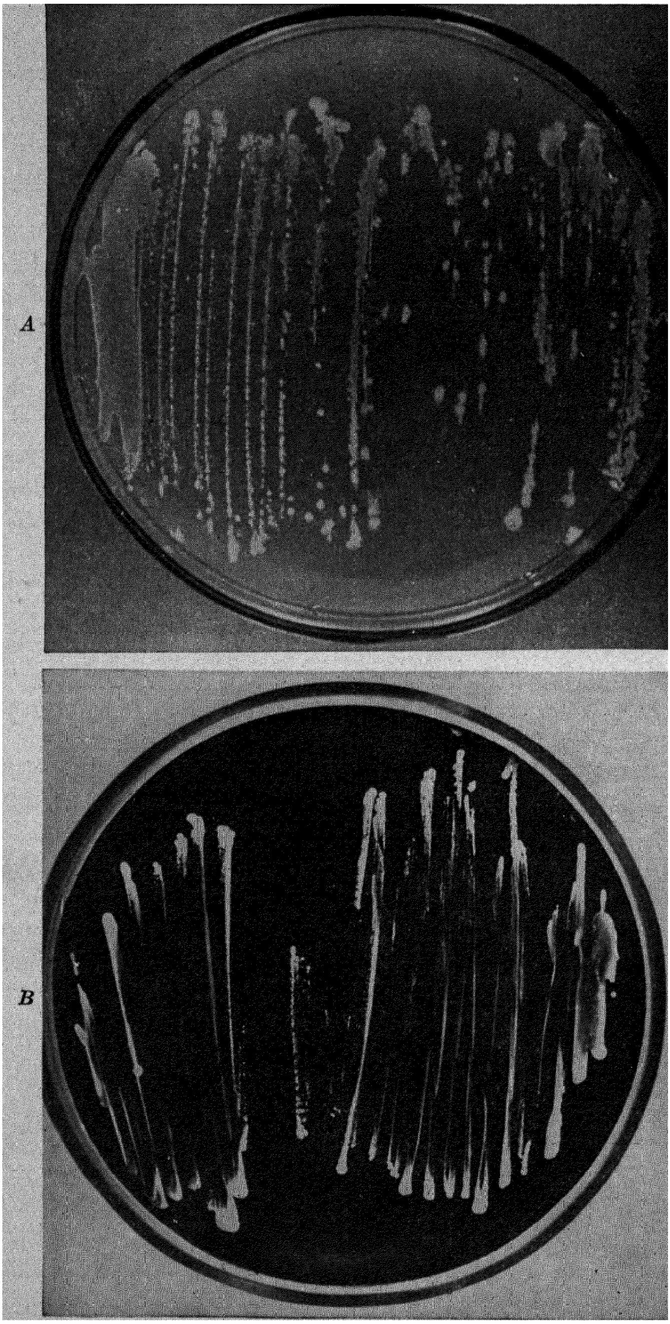

FIG. 123.—Starch hydrolysis in the presence of bacteria. *A*, *Bacillus subtilis*, no color with iodine indicating complete hydrolysis; *B*, *Escherichia coli*, blue color with iodine indicating absence or incomplete hydrolysis of starch.

For an excellent discussion on the structure of the starch granule see the report by Alsberg (1938).

Liquefaction of Gelatin.—Gelatin possesses the property of forming a gel when dissolved in warm water. Since it is a protein, it can be attacked by some bacterial organisms, resulting in the loss of its jellifying property. The hydrolysis of gelatin is an enzymatic reaction. The enzyme responsible for this change is known as a gelatinase. In the presence of carbohydrates that are rapidly fermented, gelatinase is usually not produced at all or else in very minute amounts. In general,

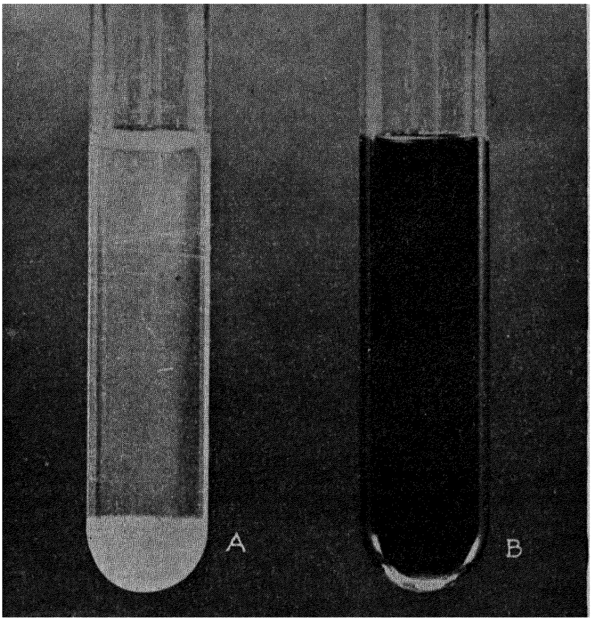

FIG. 124.—Starch hydrolysis in the absence of bacteria. *A*, *Bacillus subtilis*, no color with iodine indicating complete hydrolysis; *B*, *Escherichia coli*, blue color with iodine indicating absence or incomplete hydrolysis of starch.

noncarbohydrate media should be used to demonstrate the ability of an organism to elaborate this enzyme. Bacteria may be divided into two groups on the basis of their action on gelatin.

Hydrolysis of Casein.—Some organisms possess the power of attacking and hydrolyzing casein into compounds having smaller molecular weights. This results in the conversion of the insoluble casein into soluble products. The transformation of the insoluble casein into soluble compounds is spoken of as peptonization. The enzyme responsible for the hydrolysis of casein is called a casease.

The presence of casease can be determined by incorporating sufficient milk in an agar medium to produce an opalescent appearance. The

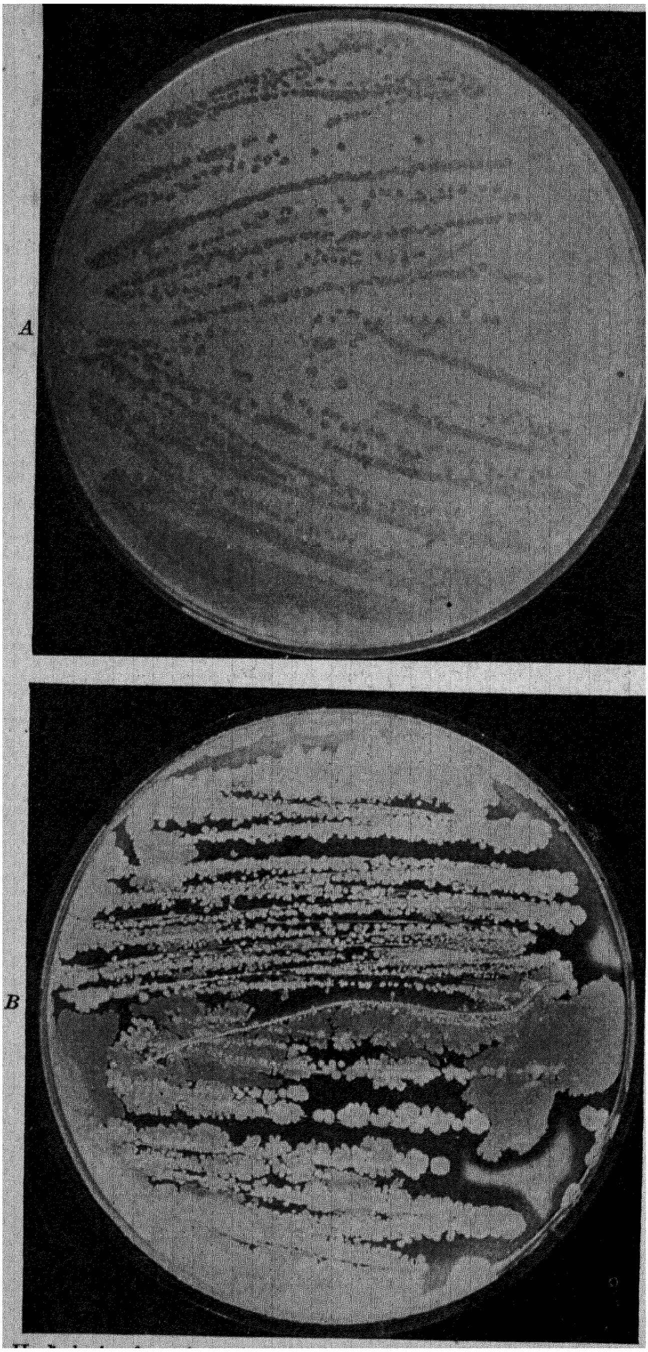

FIG. 125.—Hydrolysis of casein. *A*, *Escherichia coli* produces no hydrolysis; *B*, *Bacillus subtilis* produces a clearing of the medium, indicating hydrolysis.

organism to be tested is then streaked over the surface of the milk agar. If a culture of *Bacillus subtilis* is used, clear zones appear around each colony on the plate. This indicates that the casein has been peptonized or digested to soluble compounds by the extracellular enzyme. On the other hand, *E. coli* does not produce an extracellular caseolytic enzyme and is unable to attack the casein. Organisms can be divided into two groups on the basis of their action on milk agar.

The digestion of other compounds, such as blood (hemoglobin), starch, or inulin, may be demonstrated in a similar manner.

INTRACELLULAR ENZYMES

The intracellular enzymes are not concerned with the process of digestion but with the respiratory activities of the organism. They are not secreted outside of the cells and cannot, therefore, be demonstrated in culture filtrates. Such enzymes may be detected by employing either living, disintegrated, or dissolved bacteria. Cells may be disintegrated by making use of various types of ball mills. Suspensions of the pneumococcus and a few other species, when mixed with bile, are dissolved. The disintegrated cells and lysate may be used for demonstrating the presence of various endoenzymes in the same manner as given under the extracellular enzymes.

OXIDIZING AND REDUCING ENZYMES

The oxidation of one compound is always accompanied by the reduction of another. This involves the transfer of negative electrons from the substance oxidized to the substance reduced. For example, the reaction between ferric chloride and potassium iodide involves the oxidation of the iodine atom with simultaneous reduction of the iron atom by the transference of negative electrons from the former to the latter:

$$Fe^{+++} + 3Cl^- + K^+ + I^- \rightarrow Fe^{++} + 3Cl^- + K^+ + I$$

Many theories have been advanced to explain the mechanisms involved in intracellular oxidations and reductions. These will be discussed in Chap. XIII, under Oxidizing-Reducing Activities of Organisms, page 270.

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CHAPTER XIII

RESPIRATION OF BACTERIA

The term respiration has undergone several changes in meaning since it was first coined. Originally, respiration was used to denote the passing of air into and out of the lungs. Following this the term was extended to include the transference of oxygen to, and of carbon dioxide and water away from, the tissues. Then the term was employed to include various types of bacterial oxidations, which were referred to as aerobic and anaerobic respiration. In discussing bacterial respiration the term can be used only in its broader meaning. Therefore, any cellular reaction, whether aerobic or anaerobic, capable of yielding energy to the organism, is called bacterial respiration.

During the early years of biology it was believed that free oxygen was necessary for all organisms and that life was not possible in the complete absence of this element. Pasteur demonstrated very early in his studies that such a statement, which was then considered fundamental, had to be abandoned. He showed that there were bacteria that not only could not grow but were even killed by the presence of free oxygen. He classified bacteria as aerobic, anaerobic, facultative aerobic, and facultative anaerobic, depending upon their action toward free oxygen.

Pasteur considered all fermentation reactions, *i.e.*, the action of organisms on carbohydrates with the production of acid or of acid and gas, to be anaerobic. The escaping stream of carbon dioxide gas evolved during respiration was capable of driving the dissolved oxygen out of the media. Even though the media were exposed to the air, the conditions were anaerobic in the deeper portions. The organisms derived their oxygen from the nutrients by anaerobic decomposition.

OXIDIZING-REDUCING ACTIVITIES OF ORGANISMS

A compound may be reduced either by the addition of hydrogen or by the removal of oxygen. If one compound is reduced, another must be oxidized. Compounds that are active as hydrogen acceptors take the place of oxygen in various oxidations, being themselves reduced in the reactions. Bacterial oxidations and reductions are associated phenomena and must be studied together.

Wieland (1922) believed that almost all oxidations could be explained on the basis of the removal of hydrogen rather than on the addition of

oxygen. Hydrogen acceptors were necessary for the reactions to take place. Oxidations that occurred in this manner were called dehydrogenations. A few of the exceptions included the oxidation of aldehydes to acids, and purine bases to uric acid. In these instances Wieland believed that a preliminary hydration occurred prior to the removal of hydrogen.

1. Oxidation without preliminary hydration:

$$\underset{\text{Alcohol}}{R\cdot CH_2OH} \rightarrow \underset{\text{Aldehyde}}{R\cdot CHO} + 2H + \text{hydrogen acceptor}$$

2. Oxidation with preliminary hydration:

$$\underset{\text{Aldehyde}}{R\cdot CHO + H_2O} \rightarrow \underset{\text{Aldehyde hydrate}}{R\cdot C\begin{array}{l} \diagup H \\ \text{—}OH \\ \diagdown OH \end{array}}$$

$$\underset{\text{Aldehyde hydrate}}{R\cdot C\begin{array}{l} \diagup H \\ \text{—}OH \\ \diagdown OH \end{array}} \rightarrow \underset{\text{Acid}}{R\cdot COOH} + 2H + \text{hydrogen acceptor}$$

Theobald Smith was apparently the first to show that anaerobic, facultative anaerobic, and aerobic organisms possessed the power to reduce methylene blue. Avery and Neill (1924*a,b*) cultivated the pneumococcus (*Diplococcus pneumoniae*) under anaerobic conditions and prepared an extract of the organisms by subjecting a suspension to repeated freezing and thawing. The suspension was then centrifugated and the clear supernatant liquid passed through a Berkefeld filter in an atmosphere of nitrogen. The extract prepared in this manner was capable of producing hydrogen peroxide in the presence of oxygen and of reducing methylene blue. Heating the extract to from 55 to 60°C. destroyed the power both to reduce and to form hydrogen peroxide.

Other dyes that have been added to culture media for testing the reducing activities of bacteria include neutral red, litmus, indigo (indigo blue), and indigo carmine (sodium indigodisulfonate). Methylene blue has undoubtedly been used more than any of the other dyes for this purpose.

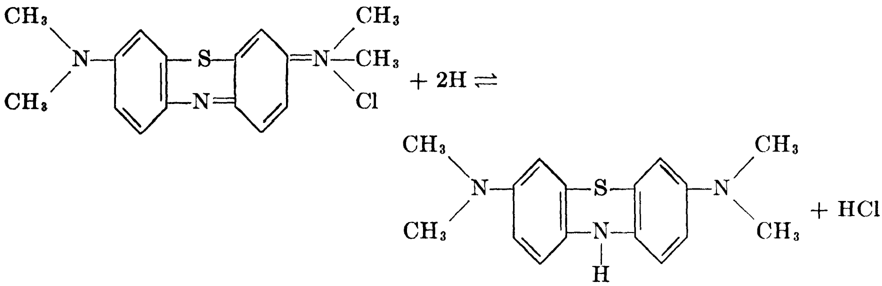
Probably all bacteria possess the power to reduce the foregoing dyes. There are, however, quantitative differences in their reducing powers. For example, *Escherichia coli* is capable of reducing methylene blue twice as fast as *Vibrio comma*, but the reverse is true for litmus.

Bacterial reductions normally occur intracellularly or at the cell surface. The evidence is not very striking to support the statement that bacterial-cell filtrates are capable of actively reducing dyes to their colorless forms. It is true that culture filtrates do possess some reducing power, which is probably due to the enzymes liberated after autolysis

of a small number of dead cells. However, the action is very mild in comparison to the vigorous reduction that occurs when filtrates of disintegrated organisms are used.

The speed of decolorization of dyes is proportional to the number of organisms present. Methylene blue is added to milk to determine quickly its approximate bacterial count. The method is of value where speed is desired. A test based on this principle is known as the reductase test (see page 449).

Reduction of Methylene Blue.—Methylene blue acts as a hydrogen acceptor and loses its color in the reduced form. If air or oxygen is bubbled through the medium containing the reduced compound, the blue color is again restored. The reaction may be represented as follows:



Methylene blue (colored) Methylene blue (colorless)

One atom of hydrogen is taken up by the double-bonded nitrogen, converting the blue-colored compound into the colorless or leuco form. The reaction is easily reversible from one form to the other.

By means of the methylene blue technique, Thunberg (1929) demonstrated the presence of respiratory enzymes in animal tissues, which were capable of activating a number of organic compounds. The methylene blue acted as a hydrogen acceptor and became reduced to the colorless or leuco form. The speed of decolorization of the dye is an indication of the rate at which oxidation takes place. The reaction must be carried out in an anaerobic environment since the presence of air quickly reoxidizes the methylene blue to the colored form.

Thunberg found that methylene blue added to suspensions of minced animal tissue was quickly decolorized because the tissue contained many substances activated by certain enzymes known as dehydrogenases. By first extracting minced tissue with water he was able to remove these substances but not the dehydrogenases. The tissue had now lost its power to reduce methylene blue. By adding various compounds to the washed tissue, Thunberg succeeded in demonstrating the presence of a large number of dehydrogenases. Such enzymes have been found to be widely distributed in higher plant and animal tissues, in yeast cells, in bacteria, etc.

Since the number of hydrogen donators is very large, it is not likely that each compound has its own specific dehydrogenase. According to Quastel (1930), the explanation that accounts most readily for the facts is that there are relatively few dehydrogenases but each enzyme deals with a particular type of molecule rather than with a specific substrate.

Dehydrogenases.—A dehydrogenase is defined as an enzyme that is capable of activating the hydrogen of metabolites. With the possible exception of uricase all dehydrogenases can act in either the presence or the absence of oxygen. Uricase appears to be an exception in that it acts only in the presence of oxygen.

The hydrogen from metabolites may be transported over several intermediate compounds, eventually combining with oxygen to produce hydrogen peroxide. The dehydrogenases are named according to the first carrier that receives the hydrogen from the substrate-dehydrogenase reaction. The dehydrogenases that are linked with carriers are generally referred to as the anaerobic dehydrogenases; those which transfer hydrogen directly to oxygen without the necessity of a carrier are called the aerobic dehydrogenases.

Aerobic Dehydrogenases.—The characteristics of the aerobic dehydrogenases, according to Potter (1939) are as follows:

1. They reduce dyes.
2. They act in the absence of oxygen if suitable dyes are present.
3. They catalyze a direct reaction between metabolites and oxygen.
4. They produce peroxide in the presence of oxygen.
5. They may or may not be inhibited by cyanide.
6. They require neither coenzyme nor cytochrome systems.

Under the aerobic dehydrogenases are included:

1. Xanthine oxidase.
2. *d*-Amino acid dehydrogenase.
3. Tyramine dehydrogenase.
4. Uricase.

Anaerobic Dehydrogenases.—The anaerobic dehydrogenases catalyze reactions between metabolites and carriers. They are differentiated according to the first carrier.

1. Coenzyme I—linked.
   a. Lactic dehydrogenase.
   b. β-Hydroxy butyric dehydrogenase.
   c. Malic dehydrogenase.
   d. Citric dehydrogenase.
   e. Alcohol dehydrogenase.
   f. Glucose dehydrogenase.
   g. Aldehyde dehydrogenase.
   h. Glyceric aldehyde phosphoric acid dehydrogenase.

2. Coenzyme II—linked.
   a. Glucose dehydrogenase.
   b. Hexose monophosphate dehydrogenase.
3. Cytochrome—linked.
   a. Succinic dehydrogenase.
   b. α-Glycerophosphate dehydrogenase.

The reactions between the dehydrogenases and their specific substrates are given on page 252 *et seq.*

Many of the dehydrogenases are differentiated by the presence of certain active groups probably attached to a common colloidal protein carrier of large molecular weight. It is difficult to believe that each dehydrogenase is a distinct enzyme with its own protein carrier. As Harrison (1935) stated,

> If each enzyme be considered distinct both as regards its active group and its colloidal carrier, it becomes difficult, in view of a large number of different substrates activated by bacteria, to imagine how so many individual enzymes, each with a very large molecule, can be accommodated in a space so small as that of the cells of bacteria.

Oxidases.—The oxidases differ from the dehydrogenases in that they appear to function by activating oxygen so that it will quickly oxidize slowly auto-oxidizable compounds (Wieland 1932). Other characteristics of the oxidases that distinguish them from the dehydrogenases are

1. They are metalloproteins (copper or iron) and are inhibited by KCN, H_2S, and other substances that stabilize the metallic groups.
2. They do not produce hydrogen peroxide.
3. They do not act under anaerobic conditions.

The following enzymes are generally classified as true oxidases:

1. Cytochrome oxidase.
2. Ascorbic acid oxidase.
3. Polyphenol oxidase.
4. Laccase.
5. Tyrosinase.
6. Dopa oxidase.

Cytochrome and Cytochrome Oxidase.—Cytochrome is a respiratory pigment, which was discovered by MacMunn (1886) but was forgotten until 1925 when it was "rediscovered" by Keilin. He named the compound cytochrome which means "cellular pigment."

The pigment is widely distributed in plant and animal cells, being found in baker's yeast, bacteria, muscles of mammals and birds, insects, molluscs and crustacea, etc. It is easily oxidized and reduced under suitable conditions. It is not a single compound but consists of three

components designated as cytochrome *a*, *b*, and *c*. The cytochrome in a yeast suspension is completely reduced by the addition of a solution of KCN. The cytochrome remains reduced because the KCN acts as an inhibitor of oxidation.

Chemically cytochrome is a hemochromogen having a structure similar to that of hemoglobin. It acts as a carrier of hydrogen. Under normal conditions, it is present in cells in the oxidized or only partly reduced form. If a suspension of yeast cells is well aerated, the cytochrome becomes oxidized; if exposed to an atmosphere of nitrogen, it becomes reduced. During conditions of great activity, the cytochrome is partly oxidized but during periods of inactivity it is again reduced.

Cytochrome oxidase is sometimes referred to as indophenolase and indophenol oxidase. The enzyme is widely distributed in nature, being found in yeasts, molds, algae, aerobic bacteria, higher plants and animals, etc.

Cytochrome oxidase is capable of oxidizing *p*-phenylenediamine to indophenol. The presence of the enzyme is easily detected by pouring a solution of *p*-phenylenediamine over the surface of an agar plate containing bacterial colonies. In the presence of the enzyme the *p*-phenylenediamine is oxidized to indophenol resulting in the appearance of a blue color. The aerobic bacteria and a few of the facultative species give a strong positive reaction; the facultative anaerobes grown aerobically give a weak positive reaction; the obligate anaerobes give a negative reaction.

Keilin (1933) reported the following properties for cytochrome oxidase:

1. It oxidizes *p*-phenylenediamine rapidly.
2. It is thermostable, being destroyed at temperatures above 60°C.
3. It is destroyed on the addition of strong alcohol or acetone.
4. Oxygen uptake during the oxidation of *p*-phenylenediamine is inhibited by the addition of minute amounts of KCN, H_2S, NaN_3, or CO.
5. *p*-Phenylenediamine does not require activation by a dehydrogenase to be oxidized by cytochrome oxidase.

Keilin and Hartree (1938*a*) stated that the only property that could be definitely ascribed to cytochrome oxidase was the oxidation of reduced cytochrome *c*. The reactions may be represented as follows:

$$\text{Metabolite}\cdot 2\text{H} + \text{dehydrogenase} + \text{cytochrome } c \rightarrow \text{reduced cytochrome } c$$
$$\text{Reduced cytochrome } c + \text{cytochrome oxidase} + O_2 \rightarrow \text{oxidized cytochrome } c$$

The reactions do not occur on the addition of KCN, H_2S, NaN_3, or CO.

For additional information on cytochrome and cytochrome oxidase see Keilin and Hartree (1939) and Schultze (1939).

Ascorbic Acid Oxidase.—Szent-Györgyi (1928) reported the isolation of a hexuronic acid from animal tissue and from orange juice. In later communications Szent-Györgyi (1930, 1931) isolated a specific enzyme from cabbage leaves which was capable of oxidizing hexuronic acid. He termed this enzyme hexoxidase. The enzyme functioned aerobically because no oxidation of hexuronic acid occurred under anaerobic conditions in the presence of dyes as hydrogen acceptors. The hexuronic acid was later shown to be identical with vitamin C, or ascorbic acid, and the hexoxidase is now referred to as ascorbic acid oxidase.

Ascorbic acid is extremely unstable, being readily oxidized to dehydroascorbic acid (see page 254). The oxidized form still displays some antiscorbutic properties. Antiscorbutic activity appears to parallel to a remarkable degree its reducing capacity. Hopkins and Morgan (1936) believed that glutathione was the chief protective substance in cells for maintaining ascorbic acid in the reduced form. It guarded the vitamin against irreversible oxidation with a loss of antiscorbutic activity. Glutathione, added to a mixture of ascorbic acid and its oxidase, maintained the vitamin in the reduced or active form.

The exact function of ascorbic acid in cellular respiration is not clearly understood. Since it is capable of alternate oxidation and reduction, it is probable that its function is that of a respiratory carrier.

Polyphenol Oxidase.—This enzyme is also known as polyphenolase, potato oxidase, phenol oxidase, etc. Polyphenol oxidase is a copper proteinate, the protein showing some difference depending upon the source of the enzyme. The oxidase is widely distributed in the plant kingdom. It is inhibited in its action by cyanide, sulfide, and carbon monoxide.

Polyphenol oxidase converts *o*- and *p*-polyhydroxy phenols to the corresponding quinones (see page 255).

Tyrosinase.—The development of a brown, orange, red, or black color around colonies of some bacteria and molds growing on agar is due to the action of tyrosinase acting on the amino acid tyrosine and related compounds. The reactions on tyrosine, according to Raper (1932), are given on page 255.

Waksman (1932), Skinner (1938), and others showed that a large number of aerobic actinomycetes were capable of producing dark-colored compounds in nonsynthetic media, and in synthetic media to which tyrosine was added. Clark and Smith (1939) noted the production of a black pigment by many strains of *Bacillus niger* (*mesentericus*) growing on several peptone agars. The addition of glucose or maltose inhibited the formation of pigment on those agars which were normally blackened by the organism. This suggested that pigment formation was due to the action of the organisms on the protein and that the addi-

tion of a fermentable carbohydrate produced a protein-sparing action. They concluded that *B. niger* produced a black pigment upon protein media, which contained free or metabolically available tyrosine. Some commercial peptones that did not contain readily available tyrosine were not blackened unless the amino acid was added. The addition of a fermentable carbohydrate to many protein media, normally blackened by *B. niger*, inhibited pigmentation unless free tyrosine was added.

For more information see Gould (1939).

Dopa Oxidase.—This enzyme is so named because of its ability to oxidize 3:4 dihydroxyphenylalanine, or dopa, to melanin. Since it is unable to oxidize many phenolic derivatives, it is considered the most specific of the oxidases so far studied. The enzyme is active only in the presence of oxygen. The addition of dopa to cells containing the enzyme results in the appearance of a black color (see page 255).

Catalase.—Some bacteria possess the power to produce hydrogen peroxide (H_2O_2) in the presence of free oxygen. Since hydrogen peroxide is toxic to living cells, its destruction is of considerable importance. The accumulation of peroxide in cultures is controlled by two factors: (1) bacterial catalase, and (2) the degree of sensitiveness of the organisms to the compound.

Catalase is an enzyme capable of decomposing hydrogen peroxide into water and molecular oxygen, according to the equation

$$2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$$

The presence of hydrogen peroxide was first detected in the pneumococcus, an organism incapable of producing catalase and only moderately sensitive to the toxic action of H_2O_2. Organisms that do not produce catalase may be protected by being cultivated with certain plant or animal tissues, or with other organisms capable of producing the enzyme.

Catalase is produced by many bacteria. Some produce more of it than others. It is present in largest amounts in the strictly aerobic bacteria. On the other hand, its presence has not been demonstrated in the obligately anaerobic bacteria.

Catalase is a chromoprotein being composed of hematin in combination with a protein. The enzyme molecule is analogous to hemoglobin in composition. The hematin appears to be identical with that present in hemoglobin but the proteins are different. The striking differences in behavior of the two compounds must be due to the characteristics of the protein components.

The concentration of catalase can be determined by adding hydrogen peroxide and noting the amount decomposed. The test is easily performed by adding peroxide to bacterial cultures contained in Smith

fermentation tubes and measuring the volumes of oxygen evolved (Fig. 126).

Peroxidase.—The recognition of peroxidase as a specific enzyme was first reported by Linossier (1898). The enzyme is widely distributed in nature, being found in vegetables, many sprouts, sugar beet, wheat flour, bran, brewer's yeast, insects, birds, animals, aerobic bacteria, milk, potatoes, etc. Horseradish is said to be the richest source of the enzyme.

Peroxidase is very resistant to heat. It is destroyed at a temperature of 98°C. in 10 min. but, on standing, as much as 25 per cent of the original activity is restored.

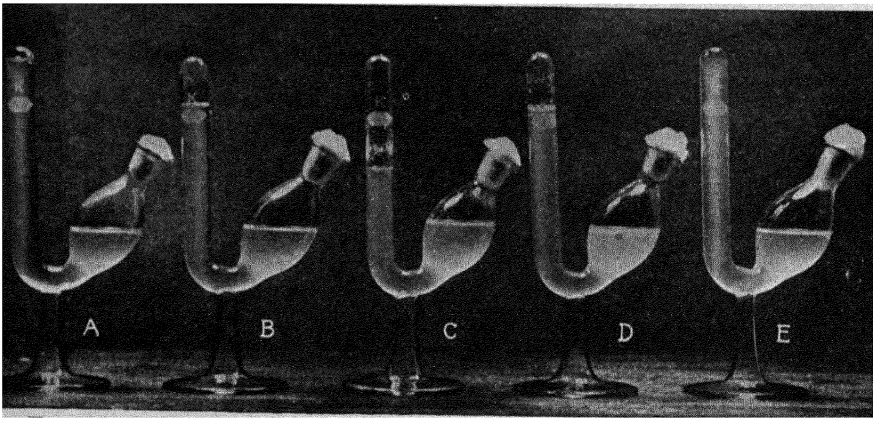


FIG. 126.—Reduction of hydrogen peroxide with the liberation of oxygen. *A, Bacillus subtilis; B, Staphylococcus aureus; C, Pseudomonas fluorescens; D, Escherichia coli; E,* control. *Bacillus subtilis* shows slight decomposition whereas *Pseudomonas fluorescens* shows vigorous decomposition. Note the absence of gas in the control.

The function of peroxidase is to transfer oxygen from peroxides to oxidizable substances. Peroxidase does not decompose hydrogen peroxide in the absence of an oxidizable substance. In this respect it differs from catalase, which decomposes hydrogen peroxide in the absence of an oxygen acceptor. The two enzymes differ also in the kind of oxygen released. Peroxidase releases atomic oxygen whereas catalase decomposes hydrogen peroxide with the liberation of molecular oxygen. A large number of compounds may function as oxygen acceptors, thereby permitting the peroxidase to act. Among these may be mentioned glutathione and cytochrome *c*. The action of the enzyme on an oxidizable substance (RH_2) in the presence of hydrogen peroxide may be represented as follows:

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{peroxidase}\cdot H_2O_2$$
$$\text{Peroxidase}\cdot H_2O_2 + RH_2 \rightarrow \text{peroxidase} + R + 2H_2O$$

The addition of KCN completely inhibits the above reactions, whereas CO has no effect.

Like catalase, this enzyme is also a compound of a hematin and a protein. The hematin present in peroxidase, catalase, and methemoglobin appears to be identical, differing only in the kind of protein with which it is combined. These compounds are similar in many respects but show considerable differences in the nature and magnitude of their catalytic activities.

Dehydrogenase-Peroxidase Systems.—Thurlow (1925) showed that when xanthine was added to xanthine dehydrogenase in the presence of a plentiful supply of air, hydrogen peroxide soon appeared in the solution. The amount present rapidly increased until a maximum was reached, after which the quantity gradually decreased owing to its reaction with the xanthine. The presence of hydrogen peroxide can be detected by the addition of peroxidase and a suitable oxidizable substance such as sodium nitrite. Peroxidase does not decompose hydrogen peroxide in the absence of an oxidizable substance. In this instance the hydrogen peroxide rapidly converts the nitrite into nitrate and the amount utilized may be determined from the unoxidized nitrite still remaining to the mixture. A typical set of results reported by Thurlow is given in Table 36. Oxidation of nitrite to nitrate occurred only in the last experiment.

McLeod and Gordon (1923*a*) and McLeod (1928) proposed a classification of bacteria on the basis of catalase and peroxide production. The classification is as follows:

Group I. The anaerobes, devoid of catalase, extremely sensitive to H_2O_2, and considered as potential peroxide producers; *Clostridium perfringens, C. tetani, C. sporogenes*, etc.

Group II. Peroxide producers devoid of catalase and only moderately sensitive to H_2O_2; the pneumococcus; many types of streptococci; the lactic acid bacteria; and some sarcinae.

TABLE 36.—OXIDATION OF NITRITE TO NITRATE

Enzyme System	Oxidation of Nitrite
Xanthine dehydrogenase + xanthine	Negative
Xanthine dehydrogenase + peroxidase	Negative
Xanthine + peroxidase	Negative
Xanthine dehydrogenase + xanthine + peroxidase	Positive

Group III. Nonperoxide producers and devoid of catalase; certain streptococci, dysentery bacilli (Shiga type), and some hemoglobinophilic bacteria.

Group IV. Bacteria producing catalase. The great majority of the bacteria capable of growing aerobically or both aerobically and anaerobically.

COENZYMES

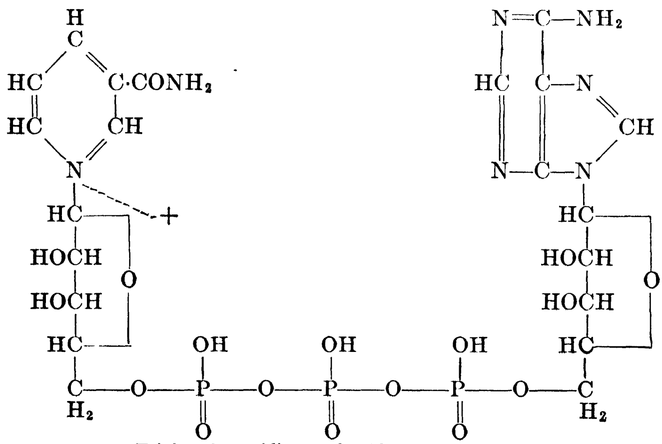
Enzymes may be defined as organic catalytic agents elaborated by living cells and capable of functioning independently of the cells. They

are heat labile, nondialyzable compounds of high molecular weight. Coenzymes are also catalytic agents produced by living cells and are necessary in enzymatic reactions, but they are heat stable, dialyzable, and have smaller molecular weights.

Coenzymes I and II.—Warburg (1932) constructed an enzyme system for the oxidation of hexosemonophosphate (Robison ester) in vitro in the presence of molecular oxygen. The system was composed of

1. Hexosemonophosphate.
2. A dehydrogenase from yeast.
3. A coenzyme from red blood cells.
4. The yellow enzyme (flavoprotein).
5. Oxygen.

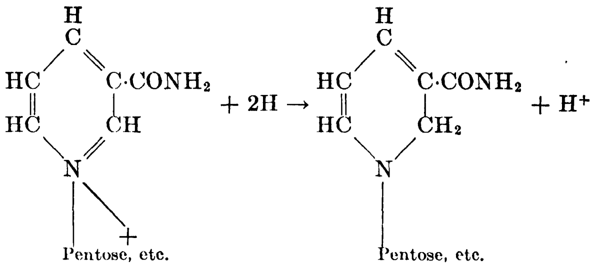
In a later communication Warburg (1935) isolated the coenzyme and characterized it as a triphosphopyridine nucleotide (TPN). It consists of one molecule of β-nicotinic acid amide, one of adenine, two of pentose, and three of phosphoric acid. He showed that the compound was capable of oxidation and reduction. The structural formula of the coenzyme is as follows:



Triphosphopyridine nucleotide (coenzyme II)

Other names for the compound are codehydrase II and coferment.

In the above system the first reaction occurred between the substrate, coenzyme II, and the specific dehydrogenase. Two atoms of hydrogen were transferred from the hexosemonophosphate to the coenzyme. The products of the reaction consisted of phosphohexonic acid and reduced coenzyme. When the coenzyme was unable to accept more hydrogen, the reaction stopped. The pyridine ring of the coenzyme acted as the hydrogen acceptor according to the following reaction:



This reaction occurred either in the presence or in the absence of oxygen. The second reaction was studied under anaerobic conditions. To the mixture of substrate, dehydrogenase, and coenzyme was added the yellow enzyme or flavoprotein. The hydrogen was now transferred from the reduced coenzyme to the flavoprotein. When both coenzyme and flavoprotein were unable to accept more hydrogen, the reaction stopped. If air was now added to the system, hydrogen was transferred from the reduced flavoprotein to molecular oxygen, which resulted in the formation of hydrogen peroxide. The reactions may be represented as follows:

$$\underset{\text{(Robison ester)}}{\text{Hexosemonophosphate}\cdot 2H} + \text{coenzyme} + H_2O \rightarrow \underset{\text{acid}}{\text{phosphohexonic}} + \text{coenzyme}\cdot 2H$$

$$\text{Coenzyme}\cdot 2H + \text{flavoprotein} \rightarrow \text{coenzyme} + \text{flavoprotein}\cdot 2H$$

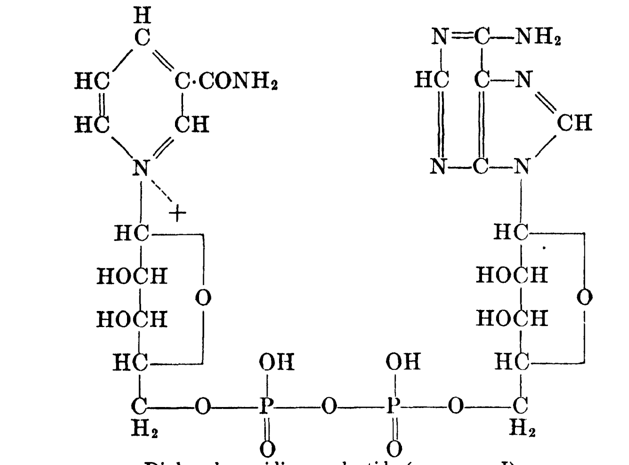
$$\text{Flavoprotein}\cdot 2H + O_2 \rightarrow \text{flavoprotein} + H_2O_2$$

The over-all reaction becomes

$$\text{Hexosemonophosphate} + H_2O + O_2 \rightarrow \text{phosphohexonic acid} + H_2O_2$$

It may be seen that the hydrogen was transported over several compounds, eventually being united with oxygen to give hydrogen peroxide. Both the coenzyme and flavoprotein were reduced and regenerated many times without being used up in the reaction. Many compounds capable of accepting hydrogen may be substituted for the flavoprotein.

Warburg isolated another coenzyme from yeast, which differed from the triphosphopyridine nucleotide in having one less molecule of phosphoric acid. This coenzyme is necessary in the fermentation of glucose to alcohol by the yeast *Saccharomyces cerevisiae* and in other reactions. Chemically the coenzyme is diphosphopyridine nucleotide (DPN). It is also called cozymase, codehydrase I, coenzyme I, yeast coenzyme, etc. The active group is also the pyridine ring, which acts as a hydrogen acceptor in the same manner as given under coenzyme II. The structural formula is as follows:

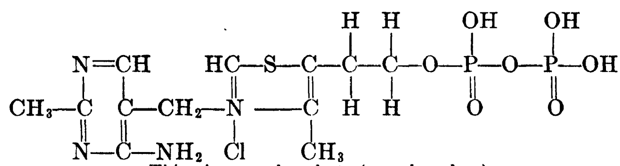


Diphosphopyridine nucleotide (coenzyme I)

Diaphorase (coenzyme factor) is one of the enzymes that has been shown to be capable of reoxidizing reduced coenzyme I. It is widely distributed in plant and animal tissues, in yeast, and in several species of bacteria. The enzyme is extremely unstable.

For more information see Lockhart (1939).

Cocarboxylase.—This coenzyme was first isolated from yeast juice. It is composed of one molecule of thiamin (vitamin B_1) and two molecules of phosphoric acid, having the following structural formula:



Thiamin pyrophosphate (cocarboxylase)

The coenzyme is necessary for the decarboxylation of pyruvic acid to acetaldehyde by the enzyme carboxylase, according to the reaction,

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + \text{carboxylase} + \text{cocarboxylase} \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + CO_2$$

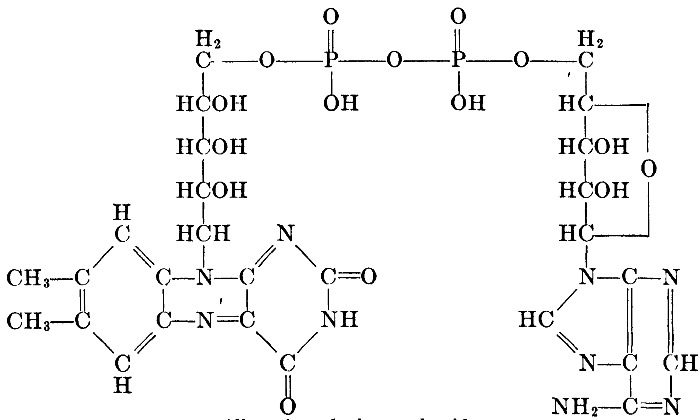
In animal tissues the coenzyme appears to be intimately connected with thiamin (vitamin B_1) and the metabolism of pyruvic acid.

Coenzyme of the d-Amino Acid Oxidase.—This coenzyme is necessary for the action of *d*-amino acid oxidase, an enzyme which is capable of oxidizing many *d*-amino acids to the corresponding keto acids. The reaction is as follows:

$$\underset{d\text{-amino acid}}{R{\cdot}CHNH_2{\cdot}COOH} + d\text{-amino acid oxidase} + \text{coenzyme} + O \rightarrow \underset{\text{Keto acid}}{R{\cdot}CO{\cdot}COOH} + NH_3$$

Warburg showed that the coenzyme is alloxazine adenine dinucleotide. The alloxazine ring of the coenzyme accepts hydrogen from the *d*-amino acid, resulting in the formation of reduced coenzyme. The reduced compound may then be reoxidized by molecular oxygen.

The following formula has been suggested for the compound:



Alloxazine adenine nucleotide

The coenzyme has been shown to be present in yeast and in many animal tissues.

Adenylic Acid.—This important coenzyme is discussed on page 330 in connection with the fermentation of glucose to alcohol by brewer's yeast.

Mode of Action of Coenzymes.—Coenzymes function by accepting hydrogen and then transferring it to other compounds until it eventually unites with oxygen. Baumann and Stare (1939*a*) stated,

> Coenzymes are no longer looked upon as accelerators in any supplementary sense, but rather as essential, integral members of a complicated "bucket brigade," transferring hydrogen or phosphate from compound to compound; they empty their buckets and come back for more. They are "carriers" of both hydrogen and phosphate. But protein enzymes of the traditional type, the dehydrogenases, must be present to bring about hydrogen transfer.

For an excellent discussion of coenzymes see the reviews by Baumann and Stare (1939*a*,*b*).

GLUTATHIONE

Hopkins (1921) isolated a thermolabile substance from extracts of yeast and muscle, which functioned as an oxidation-reduction system. The compound was found to be composed of one molecule of glutamic acid and one of cysteine united together by a peptid linkage.

In a later communication Hopkins (1929) showed that the compound was not a dipeptid, as was at first believed, but a tripeptid composed of glycine, cysteine, and glutamic acid.

The structural formula is as follows:

$$\begin{array}{rl}
\mathrm{COOH} \qquad\qquad & \mathrm{CH_2SH} \\
| \qquad\qquad\qquad & | \\
\mathrm{CHNH_2} \qquad\qquad & | \\
| \qquad\qquad\qquad & | \\
\mathrm{CH_2{\cdot}CH_2{\cdot}CO{\cdot}NH} & \mathrm{CH} \\
 & | \\
 & \mathrm{CO{\cdot}NHCH_2{\cdot}COOH}
\end{array}$$

Glutamyl cysteinyl glycine

Since it is the sulfhydryl group (—SH) which is of importance in reactions of oxidation-reduction the above formula may be abbreviated to GSH. Two molecules of reduced glutathione readily give up the hydrogen of their sulfhydryl groups and become oxidized to a disulfide.

$$\underset{\substack{\text{Reduced}\\ \text{glutathione}}}{2\mathrm{GSH}} + \mathrm{B} \rightleftharpoons \underset{\substack{\text{Oxidized}\\ \text{glutathione}}}{\mathrm{GSSG}} + \mathrm{BH_2}$$

The disulfide form is readily reduced to the original compound by the addition of two atoms of hydrogen.

Glutathione has been shown to be of almost universal occurrence in living tissue where the concentration roughly parallels the metabolic activity of the cells. The concentration is higher in rapidly growing cells than in older cells. Blood is said to contain from 34 to 47 mg. of glutathione per 100 cc. Miller and Stone (1938) demonstrated the presence of determinable amounts of glutathione in many species of aerobic and anaerobic bacteria, yeasts, and molds. *Aerobacter aerogenes* contained about 27 mg. per 100 gm.; *Proteus vulgaris*, 29 to 31 mg.; *Chromobacterium violaceum*, 7 to 45 mg.; *Monilia sitophila* (mold), 20 to 38 mg.; *Saccharomyces cerevisiae* (yeast), 59 mg.

Since the oxidation-reduction mechanism of glutathione is due to the cystine-cysteine combination, it has been shown that cystine functions in a similar manner. This may be shown by the following equation:

$$\underset{\text{Cystine}}{\begin{array}{lcl}
\mathrm{COOH} & & \mathrm{COOH} \\
| & & | \\
\mathrm{CHNH_2} & & \mathrm{CHNH_2} \\
| & & | \\
\mathrm{CH_2} & \text{—S—S—} & \mathrm{CH_2}
\end{array}} + 2\mathrm{H} \rightarrow 2\underset{\text{Cysteine}}{\begin{array}{l}
\mathrm{COOH} \\
| \\
\mathrm{CHNH_2} \\
| \\
\mathrm{CH_2SH}
\end{array}}$$

OXIDATION-REDUCTION POTENTIALS

Oxidation-reduction potentials are of great importance in biology. Reactions that occur intracellularly and release energy to the organism involve a study of oxidation-reduction potentials. An oxidation occurs

either by the addition of oxygen or by the removal of hydrogen. The oxidation of one compound involves the reduction of another.

An atom consists of a nucleus of positive electricity surrounded by a shell of electrons possessing negative electrical charges. The sum total of the negative charge must be equal to the positive charge of the nucleus. Some elements easily lose electrons whereas others add electrons. An oxidation involves the loss of one or more electrons and a reduction a gain. This may be shown in the following equations:

Oxidation:

$$Cu^{+} - \text{one electron } (\text{-le}) \rightarrow Cu^{++}$$

Reduction:

$$Cu^{++} + \text{le} \rightarrow Cu^{+}$$

A substance that readily gives up electrons is a good reducing agent; conversely, a substance that readily takes up electrons is a good oxidizing agent.

A typical oxidation by the addition of oxygen involves the formation of cupric oxide in the reaction,

$$2Cu + O_2 \rightarrow 2CuO$$

The conversion of alcohol to aldehyde is an example of an oxidation by the removal of hydrogen,

$$CH_3{\cdot}CH_2OH \rightarrow CH_3{\cdot}CHO + 2H$$

Still other oxidations occur in which oxygen or hydrogen is not involved. Iron may have a valence of three or two. The former is regarded as more highly oxidized than the latter. The oxidation of ferrous chloride to ferric chloride may be represented by the equation,

$$FeCl_2 + Cl \rightleftharpoons FeCl_3$$

This reaction involves the transfer of an electron from the iron to the chlorine atom. The same is true of all oxidation reactions. The oxidized atom loses one or more electrons to some other atom.

The transfer of electrons from one compound to another sets up a potential difference between the reactants, which may be measured by an appropriate instrument. The magnitude of this potential difference depends upon the ease with which the electrons are lost or gained. The greater the oxidizing or reducing power of a substance, the greater will be the electrical potential on one side or the other of a zero point. The more highly oxidized a substance the more positive will be its electrical potential, and the more highly reduced a substance the more negative will be the electrical potential. The direction in which a reaction proceeds is dependent upon the free electrons in the system. If the

number of electrons is increased, the system will produce more of the reductant; if the number is reduced, the system will produce more of the oxidant. The electronic state of the system is a measure of its oxidizing or reducing power.

The oxidation-reduction potential of a system is expressed by the symbol Eh. The greater the proportion of reduced substance present the lower will be the Eh value, and conversely, the greater the proportion of oxidized substance the higher will be the Eh value. When the concentration of the oxidant is equal to that of the reductant, the term becomes zero and the observed potential is equal to Eo.

ANAEROBIOSIS

Anaerobes are usually defined as organisms that can live and multiply only in the complete absence of oxygen. This statement is not strictly correct as it has been shown that all obligately anaerobic bacteria can tolerate some free oxygen. However, they show considerable variation in the amount of free oxygen they are able to tolerate. *Clostridium tetani* can grow in a liquid medium exposed to a gaseous environment containing from 10 to 20 mm. of air pressure and *C. chauvoei* can tolerate 40 mm. On a blood agar plate, *C. tetani* was found to grow in 5 to 15 mm. of air pressure. *C. perfringens* was able to produce good growth in an atmosphere containing 200 mm. of air pressure and produced a slight growth at 380 mm. of air pressure.

Mechanism of Oxygen Inhibition.—The mechanism involved in the inhibition of growth of anaerobes in the presence of molecular oxygen has been the subject of many investigations. The most important theories appear to be the following:

1. Oxygen is directly toxic to the cell.
2. Hydrogen peroxide is produced and, since the organisms do not elaborate the enzyme catalase, the compound is toxic to the cells.
3. The growth of anaerobes is dependent upon a low oxidation-reduction potential, which is not possible in the presence of free oxygen.

1. If it is true that oxygen is toxic to bacteria, growth should not take place when the organisms are transferred to a favorable environment. Such is not the case, however. Growth is reestablished when anaerobic cultures in contact with air are again exposed to a reduced oxygen environment.

2. McLeod and Gordon (1923*b*) suggested that anaerobes produced small amounts of hydrogen peroxide when exposed to air and, since they are extremely sensitive to the compound, its presence prevented the organisms from growing. This statement was based on certain observations made in connection with the growth of anaerobes in a blood medium. They observed that anaerobic organisms produced a zone of greenish

discoloration about 1/8 in. below the surface of tubes of heated blood (chocolate) agar. The growth was very similar to that produced by the pneumococcus on the same medium, which is known to be a peroxide producer.

Avery and Morgan (1924), working with several strains of pneumococcus, *Streptococcus pyogenes*, and *Diplococcus mucosus*, arrived at the same conclusions. They found that conditions favoring the formation and accumulation of peroxide in broth cultures of the above organisms were free access of air and the absence of catalase, peroxidase, and other catalysts capable of decomposing the compound. In the absence of free oxygen, peroxide was not formed. In the presence of a suitable catalyst, peroxide did not accumulate when the organisms were cultivated under aerobic conditions.

It is known that the function of catalase is to decompose hydrogen peroxide into water and molecular oxygen. Although catalase was unable to promote the growth of anaerobic organisms in contact with air, the addition of an extract rich in catalase raised the level of growth in deep tubes of agar almost to the surface. Also, the appearance of a green-colored ring in chocolate agar cultures of anaerobes was greatly delayed and decreased by the addition of catalase.

It is exceedingly difficult to demonstrate peroxide production by anaerobes since their active life is inhibited by exposure to air before sufficient peroxide has accumulated in cultures to give a positive test. It was shown that colonies of *Clostridium botulinum* appearing on the surface of blood agar plates, previously treated with benzidine, developed dark halos within an hour after exposure to air. This test indicated the production of peroxide in the presence of oxygen. Since obligate anaerobes did not produce catalase, the organisms were unable to destroy the toxic compound.

Bacteria that produce peroxide have active reducing mechanisms. A reducing mechanism is necessary for bringing active hydrogen in contact with oxygen resulting in the formation of hydrogen peroxide. All bacteria that are active reducers and devoid of catalase produce peroxide or the greenish discoloration in chocolate agar. The amount of catalase produced by different species varies depending upon the sensitiveness of the organisms to the compound.

Broh-Kahn and Mirsky (1938), in their studies on anaerobiosis, did not agree with the findings and conclusions of McLeod and Gordon. They destroyed the aerobic mechanism of *Escherichia coli* by placing the organisms in a medium containing cyanide. The respiratory system of the cyanide-poisoned organisms was replaced by the addition of a reversible dye system of suitable potential. Under these conditions the

organism could no longer tolerate free oxygen and behaved as an obligate anaerobe. To quote from their work,

. . . it is demonstrated that *E. coli*, in the absence of oxygen consumption, is unable to form peroxide. It may, therefore, be assumed that oxygen consumption must precede peroxide formation. . . . To produce the amounts of peroxide found necessary to inhibit growth, amounts of oxygen more than sufficient to be detected manometrically must be consumed. Yet obligate anaerobes have never been found to consume any amount of this gas [Fujita and Kodama (1934); Strickland (1935)]. In the case of such organisms as the pneumococcus and of *E. coli* growing in the presence of cyanide and dye, large amounts of oxygen consumption and peroxide formation may be detected and inhibition may properly be attributed to this factor. In the former organism, the relative tolerance towards H_2O_2 allows growth to proceed until amounts of peroxide sufficient to inhibit have accumulated.

3. Quastel and Stephenson (1926) believed that anaerobic growth was dependent upon a low oxidation-reduction potential, which was not possible in the presence of free oxygen. This is generally believed to be the most important reason why anaerobes fail to grow in the presence of free oxygen. Since cyanide-treated *E. coli* multiply readily in the presence of air, it appears doubtful that growth of anaerobes depends entirely upon a proper oxidation-reduction potential.

It may be concluded that no theory takes into consideration all the facts to explain why anaerobes fail to grow in the presence of free oxygen.

For more information consult the reports of Broh-Kahn and Mirsky (1938), Commoner (1940), and Vennesland and Hanke (1940).

METHODS EMPLOYED FOR THE CULTIVATION OF ANAEROBIC BACTERIA

Reduction by Heat.—Tubes of deep broth may be effective for the cultivation of anaerobes. The medium is first heated in an Arnold sterilizer to 100°C. and maintained at this temperature for 10 min. to drive out as much of the dissolved oxygen as possible. The broth is allowed to cool, without disturbing, to a temperature of 50°C. and then inoculated before there is opportunity for redissolution of oxygen from the atmosphere.

Addition of Reducing Compounds.—The addition of reducing compounds, such as glucose, cysteine, sodium formate, and sodium thioglycollate, to liquid media usually permits the growth of anaerobes under aerobic conditions. Heavy inocula are more effective than light because of the transfer of reducing substances to the new medium.

Increasing the pH of the Medium.—An increase in the pH results in an increase in the reducing intensity of the medium. The anaerobic conditions are poor but may permit the growth of those anaerobes which are less exacting in their requirements.

Use of Aerobic Bacteria.—A tube of deep agar, containing an appropriate carbohydrate, is melted and poured into a Petri dish. When the agar has solidified, the plate is placed in an incubator until the surface is free from droplets of moisture. The plate is divided into two parts by making a heavy line on the bottom of the dish with a china marking pencil. One-half of the plate is streaked with a culture of an obligate anaerobe; the other half is streaked with a culture of a facultative aerobic organism. The cover is replaced and the edges of the two halves carefully sealed with modeling clay. The plate is incubated in an inverted position.

The facultative aerobic organism utilizes the free oxygen and eliminates carbon dioxide. The oxygen tension is soon reduced to a level that permits growth of the anaerobe. Colonies of the anaerobic organism should appear in from 24 to 48 hr.

Addition of Living or Dead Tissue.—Pieces of kidney, liver, etc., removed aseptically from an animal or minced and heat-sterilized beef heart or brain tissue have been used for the cultivation of obligate anaerobes.

Minced and sterilized brain medium has been used probably more than any other tissue preparation for the cultivation of anaerobes. It is prepared by suspending cooked, minced sheep brains in glucose broth. The medium is heated for about 10 min. in an Arnold sterilizer just before inoculation to drive out as much of the dissolved oxygen as possible. The tubes are allowed to cool to a temperature of about 50°C., then inoculated by loop or pipette plunged deep. A surface seal is not necessary because the minced brain tissue is very efficient in increasing the reducing power of the medium. Aerobes grow throughout the medium and anaerobes multiply in the deeper portions (Fig. 127).

Exclusion of Atmospheric Oxygen.—A tube of deep nutrient broth or an appropriate carbohydrate broth is inoculated and then covered with a ½-in. layer of melted sterile vaspar (a mixture of equal parts of vaseline and paraffin). It does not prevent entirely the entrance of atmospheric oxygen, but it is usually sufficient in establishing initial growth. After growth has once set in, the elimination of carbon dioxide by the organisms creates optimum conditions for multiplication. It is advisable to heat the media in an Arnold sterilizer for 10 min. previous to inoculation in order to drive out as much of the dissolved oxygen as possible.

Agar may be substituted for the broth. The medium is melted and cooled to about 50°C. The agar is inoculated and thoroughly mixed by gentle shaking and rotation. The medium is allowed to set in a vertical position, after which the surface is covered with a ½-in. layer of melted vaspar.

The value of this method is that it affords a simple means of grading the oxygen tension in the medium. On the surface the pressure is atmospheric whereas at the bottom the conditions are anaerobic. The agar cylinder is removed by cutting the tube in the center, pulling the two halves apart, and collecting the agar in a sterile Petri dish. The colonies can then be fished from the agar and examined.

Semisolid Agar Medium.—An appropriate medium containing from 0.05 to 0.2 per cent of agar is sometimes sufficient in establishing growth of anaerobic organisms. The medium is either liquid or semisolid,

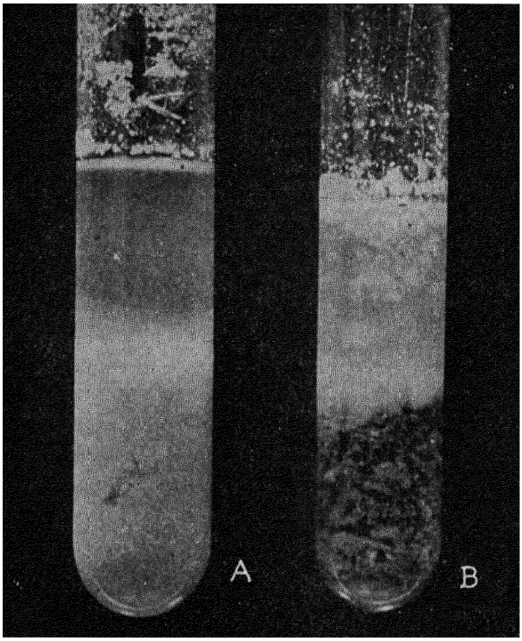


FIG. 127.—Anaerobic cultures of *Clostridium sporogenes*. *A*, minced brain medium; *B*, beef heart medium.

depending upon the concentration of agar employed. The agar lessens convection currents, thereby prolonging anaerobic conditions in the medium.

Replacement of Atmospheric Oxygen with Hydrogen.—In this procedure the inoculated tubes are placed in a closed jar. The air is evacuated and replaced with hydrogen gas. The jar is then placed in an incubator.

This procedure does not always prove satisfactory, owing to the fact that sufficient oxygen usually remains in the medium to prevent growth. The method becomes considerably more efficient if an alkaline solution of pyrogallol is added to the jar just before the air is removed.

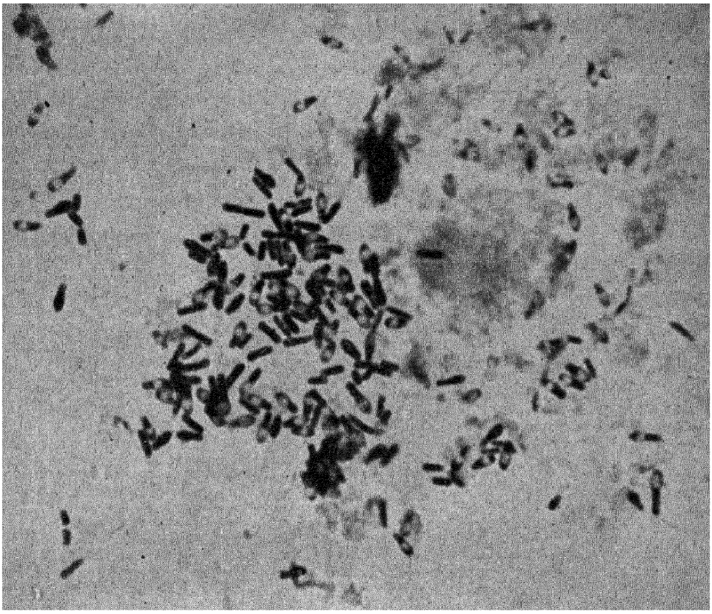


FIG. 128.—Bacilli and spores of *Clostridium botulinum*.

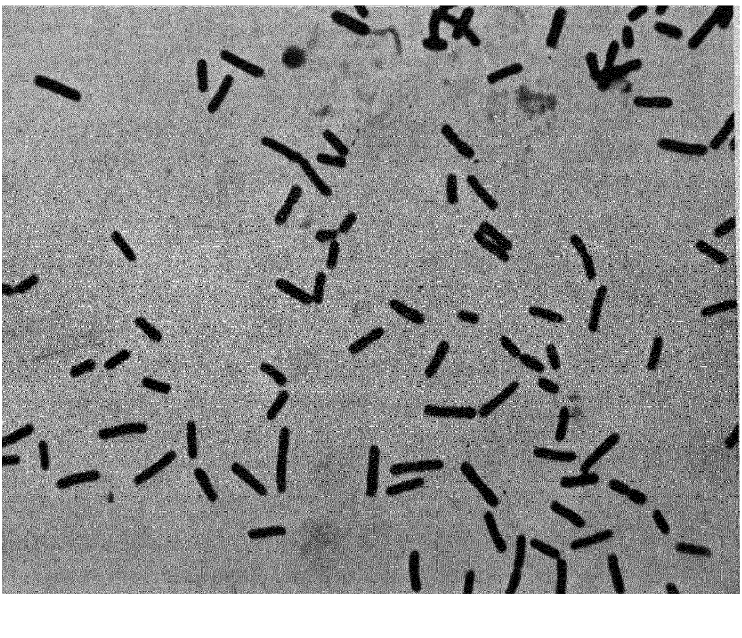


FIG. 129.—*Clostridium perfringens* (*welchii*).

This is best performed by placing some pyrogallol crystals on the bottom of the jar, followed by the addition of sufficient solution of potassium hydroxide to dissolve the compound. The lid is quickly replaced and the air removed as quickly as possible. The pyrogallol usually removes any oxygen still remaining in the medium, permitting growth of the strict anaerobes.

Combustion of Oxygen with Hydrogen.—Surface colonies are most essential for isolating and identifying anaerobes. The methods already described are not entirely satisfactory for this purpose.

The removal of oxygen by combustion with hydrogen is probably the most efficient method for obtaining surface colonies of anaerobic organisms. For a description of the apparatus and method of operation see the report of Fildes (1931).

REDUCTION OF NITRATES

Many bacterial species are able to reduce nitrates to nitrites and finally to ammonia. Some species are capable of reducing nitrates to nitrites but cannot reduce nitrites to ammonia. Still others are unable to attack nitrates but can reduce nitrites to ammonia. The nitrates support anaerobic growth by acting as hydrogen acceptors. An example of such a reaction is the oxidation of lactic acid to pyruvic acid under anaerobic conditions in the presence of potassium nitrate.

$$\underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} + KNO_3 \rightarrow \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2O + KNO_2$$

The potassium nitrate accepts hydrogen, being itself reduced to potassium nitrite.

The reduction of nitrates proceeds more rapidly in the presence of an anaerobic or partial anaerobic environment. Organisms capable of reducing nitrates and nitrites are well distributed in nature. It has been shown that many strict aerobic forms can live under anaerobic conditions in the presence of nitrates. If the culture is well aerated, nitrate reduction does not occur. The reaction of the medium is of importance in determining the extent of reduction. In an alkaline medium the nitrates are reduced to nitrites, whereas in an acid environment the reaction may proceed to the ammonia stage.

Certain organisms are capable of reducing nitrates to ammonia, with nitrites produced as an intermediate compound. A few species can produce nitrogen from nitrates. The organisms are capable of growing in the presence of oxygen but reduce nitrates only under anaerobic conditions. An increase in the oxygen supply causes a corresponding decrease in nitrate reduction.

In making tests for nitrate reduction observe for (1) the reduction of nitrate to nitrite, (2) the disappearance of nitrite to ammonia, and (3) the presence of nitrogen gas. It is highly important that tests be made for these three products.

The presence of nitrite indicates that the nitrate has been reduced. The presence of gas shows that the nitrite has been reduced to ammonia and finally to nitrogen. Negative results may mean that (1) the organism in question is unable to reduce nitrate or (2) the medium is not satisfactory for growth. The medium may be improved by increasing or

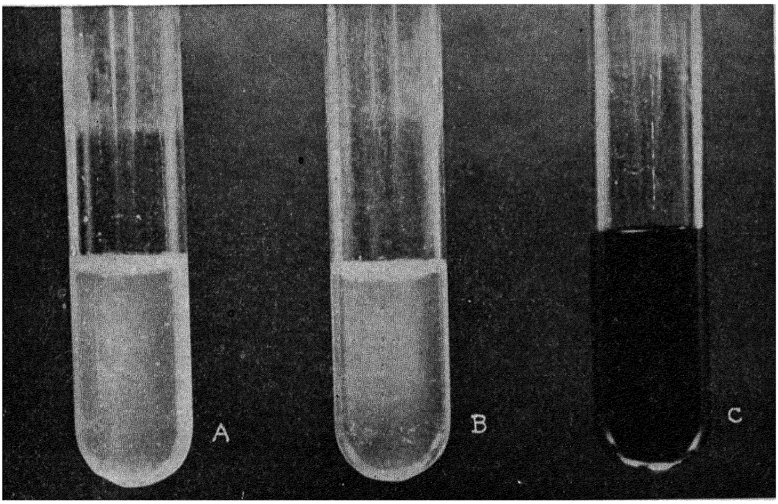


FIG. 130.—Reduction of nitrate to nitrite. *A*, control; *B*, ***Pseudomonas fluorescens***, no reduction; *C*, *Proteus vulgaris*, reduction (red color with sulfanilic acid-α-naphthylamine test solution).

decreasing the amount of peptone; by increasing the concentration of nitrate; by adjusting the reaction of the medium to a more favorable pH; by adding a readily available carbohydrate to stimulate growth; or by incorporating a small amount of agar to increase the viscosity of the medium.

The nitrite test may be negative even though good growth has occurred. This may indicate no action on the nitrate or complete reduction of the compound beyond the nitrite stage. Therefore, tests for nitrate should be made in every case where a negative nitrite test is obtained. Diphenylamine and concentrated sulfuric acid should be used for the test. A blue color indicates the presence of nitrate. Since nitrite gives the same reaction, the test should not be performed in the presence of this compound. If none of these tests shows nitrate reduction, the organism is probably not capable of attacking nitrate.

REDUCTION OF SULFATES

Beijerinck (1895) was the first to show that the hydrogen sulfide found in mud was produced by the anaerobic reduction of sulfates. Van Delden (1904) obtained pure cultures of the sulfate-reducing organism which he named *Vibrio desulfuricans.*

Van Delden's medium was composed as follows:

Gelatin	10 gm.
Sodium lactate	0.5 gm.
Asparagine	0.1 gm.
$MgSO_4 \cdot 7H_2O$	0.1 gm.
K_2HPO_4	0.05 gm.
Ferrous ammonium sulfate	trace
Tap water	1000 cc.

The organisms obtained their energy from the anaerobic reduction of the sulfate, accompanied by a simultaneous oxidation of the lactate. The proportion of carbonic acid and hydrogen sulfide produced was in the ratio of 2 : 1. From this observation van Delden considered the reaction to proceed as follows:

$$2CH_3 \cdot CHOH \cdot COONa + 3MgSO_4 \rightarrow 3MgCO_3 + Na_2CO_3 + 2CO_2 + 2H_2O + 3H_2S$$

In the presence of sodium malate instead of lactate the following reaction occurred:

$$2COONa \cdot CH_2 \cdot CHOH \cdot COONa + 3MgSO_4 \rightarrow 3MgCO_3 + 2Na_2CO_3 + 3CO_2 + H_2O + 3H_2S$$

In this reaction the molecular proportion between carbonic acid and hydrogen sulfide is about 8:3.

A general equation for the reduction of sulfates may be represented as

$$2C + MeSO_4 + H_2O \rightarrow MeCO_3 + CO_2 + H_2S$$

in which C represents the organic substrate and Me a metal.

Baars (1930) showed that a large number of organic compounds could be substituted for the lactate or malate, all of which were oxidized in an appropriate sulfate medium inoculated with *Vibrio desulfuricans.* The organic compounds are oxidized by the removal of hydrogen. In the other words, they act as hydrogen donators. A few typical examples are the following:

$$\underset{\text{Formic acid}}{H \cdot COOH} \rightarrow CO_2 + 2H$$

$$\underset{\text{Acetic acid}}{CH_3 \cdot COOH} + 2H_2O \rightarrow 2CO_2 + 8H$$

$$\underset{\text{Lactic acid}}{CH_3 \cdot CHOH \cdot COOH} + 3H_2O \rightarrow 3CO_2 + 12H$$

$$\underset{\text{Glycerol}}{CH_2OH \cdot CHOH \cdot CH_2OH} + 3H_2O \rightarrow 3CO_2 + 14H$$

In the reduction of sulfate each molecule required 8 atoms of hydrogen for its conversion into hydrogen sulfide. The dehydrogenation of the organic substrate proceeded by steps followed by a corresponding hydrogenation of the sulfate to produce hydrogen sulfide.

$$H_2SO_4 + 8H \rightarrow H_2S + 4H_2O$$

It was shown that unless the organic compound was present in large excess complete oxidation did not occur. Van Delden's medium contains 15 millimols of sulfate per liter. From this it follows that 10 millimols of lactate should be sufficient to produce complete reduction of the sulfate. In actual experiment, however, it was found that 30 millimols were required for complete oxidation. The reaction for the incomplete oxidation was

$$2CH_3\cdot CHOH\cdot COONa + MgSO_4 \rightarrow 2CH_3\cdot COONa + MgCO_3 + CO_2 + H_2O + H_2S$$

Organism.—*Vibrio desulfuricans* is an obligate anaerobe, which produces spores under natural conditions but fails to do so on laboratory media. The organisms can be isolated by inoculating soil or mud into media containing 0.5 per cent sodium sulfate and incubating the culture under anaerobic conditions. A loopful of the liquid culture is transferred to a sulfate agar plate containing an iron salt. After incubation the colonies of *V. desulfuricans* can be easily recognized by the fact that the sulfide-producing organisms react with the iron salt to produce black iron sulfide.

CARBON DIOXIDE REQUIREMENT OF BACTERIA

It is well known that certain photosynthetic bacteria utilize carbon dioxide in the presence of water and produce organic matter (page 514). It has been shown that carbon dioxide is also necessary for the growth of the nonphotosynthetic bacteria. The *Propionibacterium* require carbon dioxide for the production of propionic and succinic acids (page 338). Since bacteria produce carbon dioxide as a metabolic end product, it seems strange that it is also required for growth.

Wherry and Ervin (1916) noted that if the carbon dioxide evolved by the tubercle bacillus was removed as rapidly as it was formed growth failed to occur. They believed that an accumulation of a definite amount of the gas was necessary before growth took place. Chapin (1918) and Cohen and Fleming (1918) showed that the gas was necessary for the growth of the gonococcus and the meningococcus. Huddleson (1921) reported that the growth of *Brucella abortus* was stimulated by the presence of carbon dioxide. Rockwell (1921, 1923) and Rockwell and Highberger (1926) showed that certain anaerobes were unable to grow in a carbon dioxide-free environment. They found that many organisms,

which failed to grow in an atmosphere of pure nitrogen, showed active multiplication on the addition of carbon dioxide. Valley and Rettger (1925) found that many bacteria grew better in the presence of an increased concentration of carbon dioxide. Kulp (1926) obtained similar results. Theobald Smith (1926) verified the findings of Huddleson with respect to growth stimulation of *B. abortus*. A larger inoculum resulted in an increased tendency to obtain growth. He believed that an increased number of organisms favored a more rapid accumulation of carbon dioxide, which resulted in growth. McAlpine and Slanetz (1928) increased the concentration of the gas to from 5 to 10 per cent and found that the growth of *B. abortus* was greatly stimulated. The organisms showed a decreased growth in the complete absence of the gas. Many other examples giving similar results may be cited from the literature.

In the light of the above results the statement that carbon dioxide is a useless, toxic waste product can no longer be considered true.

For more information see the excellent reports by Foster, Carson, Ruben, and Kamen (1941), and van Niel, Ruben, Carson, Kamen, and Foster (1942).

Variation in CO_2 Production with Age.—Young cells show a much higher rate of metabolism than old cells. Huntington and Winslow (1937) reported that carbon dioxide production of 1-hr.-old bacteria was from 3 to 14 times higher than that of 24-hr.-old cells. The peak of CO_2 production per cell was reached in from 2 to 5 hr. after inoculation of old cells into fresh medium. Rahn (1941) reported similar results. The removal of CO_2, by absorption in a solution of KOH, did not prevent growth of young cells unless the number per drop was very small. When a drop showed less than 10 organisms, growth did not occur in the absence of CO_2.

Effect of Large and Small Inocula.—Rahn showed that heavy cell concentrations produced CO_2 more rapidly than it could be removed, with the result that the bacteria developed even though potassium hydroxide was present to absorb the gas. Light cell concentrations, on the other hand, failed to produce sufficient gas in the presence of KOH to permit growth. The probability of growth was lessened as the number of cells per drop decreased. The fact that a large inoculum produces more carbon dioxide than a small one would seem to afford an explanation for the shorter lag phase with larger inocula (see page 226).

OXYGEN REQUIREMENT OF BACTERIA

It may be stated that all living plants and animals consume oxygen and eliminate carbon dioxide. Most bacteria also utilize oxygen (either free or combined) and eliminate carbon dioxide. A few bacterial species

have been studied that apparently do not eliminate carbon dioxide. Rahn (1941) reported that *Lactobacillus acidophilus*, *L. casei*, and *Streptococcus lactis*, in young or old cultures, did not produce carbon dioxide. However, carbon dioxide was necessary for growth, even though the organisms failed to produce the gas.

Rahn and Richardson (1940) reported that the oxygen supply of bacteria in the usual cultural methods was far from adequate. As an example, the tubercle bacillus requires 1.22 per cent oxygen for the complete oxidation of 1 per cent of glycerol. The medium, at 37°C., contains only 0.00065 per cent of oxygen. The disproportion between supply and demand is so great that a majority of the cells in a culture probably starve for lack of oxygen.

Rahn and Richardson measured the oxygen consumption of multiplying bacteria per cell per hour. The results are recorded in Table 37. The consumption of oxygen does not depend entirely on cell size. Pseudomonas required more oxygen than *E. coli* for the same amount of cell substance.

TABLE 37.—OXYGEN CONSUMPTION FOR A NUMBER OF BACTERIAL SPECIES

Organism	Oxygen Consumption per Cell per Hr., 10^{-10} Mg.
Streptococcus liquefaciens	7.22
Streptococcus faecalis	2.32
Streptococcus durans	1.07
Streptococcus lactis 125	0.46
Streptococcus lactis L21	2 10
Escherichia coli	3 9
Escherichia coli var. *communis*	3 0
Aerobacter cloacae	4.0
Aerobacter aerogenes	3.3
Proteus vulgaris	5.3
Pseudomonas fluorescens	9.3
Pseudomonas aeruginosa	8.4
Bacillus subtilis	20.6
Bacillus cereus	32.4
Bacillus mesentericus	9.5
Bacillus megatherium	65.0
Bacillus peptogenes	82.4

VARIATIONS IN OXYGEN AND CARBON DIOXIDE TENSIONS

Organisms differ widely in their sensitiveness to free oxygen. Berghaus (1907) determined the maximum tension of oxygen required to kill bacteria and also the amount that just permitted growth. *Staphylococcus* was the least affected by an increase in the oxygen tension, being

capable of withstanding a pressure of 3 atm. *Alkaligenes faecalis, Shigella dysenteriae, Pseudomonas aeruginosa,* and *Vibrio comma* were prevented from growing by slight increases in the oxygen concentration, but they showed considerable variation in the amount necessary to cause death. For example, 2 atm. of oxygen killed *V. comma,* whereas 75 atm. was necessary to destroy *P. aeruginosa.*

Moore and Williams (1911) tested 26 organisms in an atmosphere containing 500 mm. or more of oxygen. *Mycobacterium tuberculosis* and *Pasteurella pestis* were the only organisms that were inhibited in growth. Karsner, Brittingham, and Richardson (1923) used atmospheres containing from 80 to 99 per cent of oxygen and found that *Staphylococcus aureus, Escherichia coli,* and other organisms were sensitive to such concentrations. Novy and Soule (1925) found that the organisms of human and bovine tuberculosis were stimulated in growth by oxygen concentrations of from 40 to 60 per cent but were inhibited by an atmosphere containing 80 per cent or more of the gas.

Aerobic organisms inoculated into favorable culture media will grow in diminished oxygen tensions but the rate of multiplication will be less than under ordinary atmospheric conditions. Entirely different results were obtained when such organisms were cultivated under slight or extensive starvation. Cahn-Bronner (1940) cultivated *Serratia marcescens, P. aeruginosa, E. coli, Eberthella typhosa, Salmonella schottmuelleri, Shigella dysenteriae, S. paradysenteriae, Corynebacterium diphtheriae, Staphylococcus aureus* and *S. albus* under partial starvation in deep tubes of agar and found that the organisms grew in a fine, dense layer below the surface of the medium where the relation between the nutrient content and oxygen was at an optimum. Under increasing starvation (more dilute medium) the bacteria become progressively more sensitive to oxygen. The lower the concentration of carbon compounds in the medium, which may act as oxygen acceptors, the more inhibitory was the action of oxygen on the organisms.

Novy and Soule (1925) cultivated the tubercle bacillus in gas mixtures containing 30, 40, 50, 60, and 90 per cent of carbon dioxide and an adequate supply of oxygen. Luxuriant growths were obtained with the various concentrations of carbon dioxide.

RESPIRATORY QUOTIENTS OF BACTERIA

The ratio of the amount of carbon dioxide eliminated to the oxygen consumed is known as the respiratory quotient. This may be written

$$\frac{CO_2}{O_2} = R.Q.$$

On complete oxidation a carbohydrate, such as glucose, yields CO_2 and H_2O according to the equation,

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

Since the volume of CO_2 eliminated is the same as the oxygen consumed, the theoretical respiratory quotient of a carbohydrate is

$$\frac{6CO_2}{6O_2} = 1.00$$

Fat combustion is usually represented by the oxidation of palmitin as follows:

$$2(C_{15}H_{31}COO)_3C_3H_5 + 145O_2 \rightarrow 102CO_2 + 98H_2O$$

The theoretical respiratory quotient of a fat is

$$\frac{102CO_2}{145O_2} = 0.70$$

In like manner the theoretical respiratory quotient for a protein on complete combustion is 0.80.

The presence of an easily available carbohydrate gives a quotient of approximately 1.00. In the absence of an easily fermentable carbohydrate the quotient will approach that of a protein. The respiratory quotient gives an insight as to the kinds of nutrients being utilized by an organism.

Soule (1928) reported the respiratory quotients of a number of organisms grown on various types of culture media. The results are recorded in Table 38. It may be seen that the addition of glucose to media resulted in an increase in the respiratory quotients. Since the combustion of pure glucose yields a quotient of 1.00, this figure was closely approximated in several cases. Figures less than 1.00 indicated that the organisms utilized amino acids as well as glucose. In the absence of glucose the respiratory quotients approximated that of pure protein.

A factor that complicates the accuracy of the quotients is the decarboxylation of amino acids and organic acids, yielding more carbon dioxide without a corresponding utilization of oxygen. This may possibly account for those quotients greater than 1.00.

TABLE 38.—RESPIRATORY QUOTIENTS OF A NUMBER OF BACTERIA ON DIFFERENT MEDIA

Organism	Nutrient agar	Glycerol agar	Glucose agar	Serum agar
Mycobacterium tuberculosis var. *hominis*	.....	0.856	0.992	0.904
Mycobacterium tuberculosis var. *bovis*	0.888	0.903	1.036	0.852
Bacillus subtilis	0.912	0.843	1.278	0.874
Malleomyces mallei	0.841	0.859	0.972	
Theoretical value	0.810	0.857	1.000	0.810

ASSIMILATION AND RESPIRATION

Barker (1936) showed that the oxidation of carbohydrates by certain organisms, in the presence of a plentiful supply of oxygen, did not proceed to completion ($CO_2 + H_2O$) but that a portion of the substrate was assimilated by the cells. He represented the oxidative assimilation of acetate by the organism *Prototheca zopfii* as follows:

$$\underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + O_2 \rightarrow (CH_2O) + CO_2 + H_2O$$

The acetic acid was oxidized to carbon dioxide, water, and a compound having the empirical formula of a carbohydrate. Barker concluded that the oxidation of organic compounds by *Prototheca zopfii* was a process of oxidative assimilation leading to the synthesis of a carbohydrate that was stored in the cells.

Giesberger (1936) arrived at the same conclusion in his studies on three species of Spirillum. Clifton (1937) experimented with the organism *Pseudomonas calcoacetica* and obtained results similar to those reported by Barker and by Giesberger. The oxidative assimilation of acetate and butyrate by the organism was represented by the equations

$$\underset{\text{Acetate}}{2CH_3{\cdot}COOH} + 3O_2 \rightarrow (CH_2O) + 3CO_2 + 3H_2O$$

$$\underset{\text{Butyrate}}{2C_3H_7{\cdot}COOH} + 7O_2 \rightarrow 3(CH_2O) + 5CO_2 + 5H_2O$$

On the addition of suitable concentrations of iodoacetate, sodium azide, 2:4-dinitrophenol, or methyl urethane, Clifton was able selectively to poison the cells in such a way that assimilation was prevented, but not respiration, thus effecting a complete oxidation of the substrate to CO_2 and H_2O.

In the absence of the assimilatory poisons the respiratory quotient of butyrate during the stage of rapid oxidation was found to be 0.68. In the presence of the poisons (complete oxidation) the respiratory quotient was 0.80.

In a later report Clifton and Logan (1939) found that the oxidation of a number of organic compounds, including lactate, pyruvate, glycerol, and glucose, by *Escherichia coli* was not carried to the final stage of carbon dioxide and water but instead a portion of the compound was assimilated by the organisms. He postulated that the general assimilation process may be represented by the reaction,

$$\text{Substrate} + O_2 \text{ (yielding energy)} \rightarrow \text{assimilated compound} + CO_2 + H_2O$$

van Niel and Anderson (1941) conducted similar experiments on the fermentation of sugar by the yeast *Saccharomyces cerevisiae* and found that the production of equimolar quantities of carbon dioxide and alcohol

together accounted for only 70 per cent of the utilized carbohydrate. They used the expression "fermentative assimilation" to express the incomplete oxidation of the sugar. On the other hand, no such fermentative assimilation was observed during the fermentation of lactic acid by *Streptococcus faecalis*. Dextrose or levulose was rapidly and completely fermented to carbon dioxide and water.

CHROMOGENESIS

Many bacteria produce colored compounds known as pigments. This is especially true of the strictly aerobic bacteria. Many shades are produced ranging in color from red to violet.

Very little is known concerning the chemical composition of pigments. This is due largely to the fact that it is difficult to obtain sufficient pigment in a high state of purity. Some of the pigments remain confined within the bacterial cells; others are secreted into the surrounding media, giving them a characteristic appearance. Only a few of the pigments are water soluble. The majority of them are soluble in fat solvents such as alcohol, acetone, ether, and chloroform.

Pigments are probably produced under both aerobic and anaerobic conditions, but the colored form usually appears only in the presence of oxygen. When pigmented cultures are placed in an anaerobic environment, the color gradually fades until completely decolorized. On exposure of such cultures to oxygen, the color is gradually restored. Certain special media are required for strong pigment production. Solid media are better than those not containing agar. In some cases certain mineral salts are necessary. The hydrogen-ion concentration of the medium influences the color of some pigments. A temperature of 20 to 28°C. appears to be best for the production of most pigments. As the temperature increases, pigment production gradually decreases.

With few exceptions (notably the green and purple photosynthetic bacteria), pigment formation takes place best in the dark. Baker (1938) examined 185 strains of acid-fast organisms and found that 12 of them produced their characteristic pigment only in the presence of light. Pigment was entirely absent when the organisms were cultivated in total darkness.

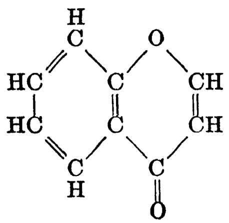
Most bacterial pigments may be classified as (1) carotenoids, (2) anthocyanins, and (3) melanins.

Carotenoids.—The carotenoids are red, orange, or yellow pigments, which are soluble in fat solvents such as alcohol, ether, or chloroform. They derive their name from the unsaturated hydrocarbon carotene, $C_{40}H_{56}$, the same coloring matter present in butter, yolks of eggs, flour, and carrots. Oxidized carotene $C_{40}H_{56}O_2$ is known as xanthophyll. The two pigments are usually found together in nature.

Some organisms that produce carotenoid pigments include the following:

1. *Sarcina lutea* produces bacterioxanthophyll.
2. *S. aurantiaca* shows the presence of β-carotene and zeaxanthin.
3. *Staphylococcus aureus* gives zeaxanthin.
4. *Mycobacterium phlei* produces lutein, kryptoxanthin, and α-, β-, and γ-carotenes.
5. *Torula rubra*, a red yeast, produces β-carotene and torulene.

Anthocyanins.—The anthocyanins include the red and blue pigments and intermediate shades found in the petals of many flowers and some bacteria. They are soluble in water and alcohol but not in ether. Most, if not all, of them are natural indicators changing usually from red in acid solutions to green in alkaline solutions. They are glucosides and on hydrolysis yield a sugar and some derivative of benzopyrilium.



Benzopyrilium

The chief derivatives are said to be pelargonidin, cyanidin, delphinidin, and oenidin.

Actinomyces waksmanii is an example of an organism that produces an anthocyanin pigment.

Melanins.—The melanins include certain brown, black, orange, and red pigments that are insoluble in almost all solvents. They are dissolved by concentrated sulfuric acid and reprecipitated by the addition of water. The pigments are produced from the decomposition of proteins by boiling concentrated mineral acids, or by the action of the enzyme tyrosinase on the amino acid tyrosine. In addition to tyrosine the enzyme attacks several other compounds, including tryptophane.

The following organisms produce melanin pigments:

1. *Azotobacter chroococcum* forms a black melanin pigment.
2. *Clostridium perfringens* is said to elaborate a black pigment.
3. *Aspergillus niger* produces a black melanin pigment known as aspergillin.
4. Many species of *Actinomyces* produce various colored melanin pigments.

Function of Pigments.—Little is known concerning the physiological functions of nonphotosynthetic pigments. Because pigment production generally takes place in the presence of oxygen, some believe that they act as respiratory carriers. Evidence available at the present time, however, is not sufficient to support this view.

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CHAPTER XIV

DECOMPOSITION AND PUTREFACTION OF PROTEINS

Proteins are extremely complex substances, which are essential constituents of all living cells, both animal and vegetable. The name protein is derived from the Greek πρωτεῖος, which means preeminence, or of first importance. They all contain carbon, hydrogen, nitrogen, and oxygen. Sulfur is found in all except certain basic proteins known as the protamines; phosphorus and iron are found in a few. The proportions of these constituents are approximately as follows: C, 51 per cent; H, 7 per cent; N, 16 per cent; O, 25 per cent; S, 0.4 per cent; P, 0.4 per cent.

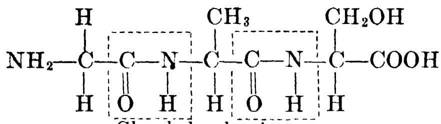
Most proteins are colloidal in nature, which means that they are composed of very large molecules. One of the properties of colloids in solution is that they are unable to pass through certain membranes, such as parchment, collodion, and animal. Most proteins form opalescent solutions—another indication that their molecules are large. They are usually amorphous but some have been obtained in crystalline form.

Another property of proteins is that when they are treated with proteolytic enzymes, or heated for some time with mineral acids, the molecules are decomposed into a mixture of compounds known as amino acids. The amino acids are sometimes referred to as the building stones of the protein molecule. The hydrolysis of proteins to the stage of amino acids results in a complete loss of colloidal characteristics.

About 20 amino acids have been recognized as constituents of protein molecules. The acids are all soluble in water with the exception of tyrosine and cystine. Tyrosine is sparingly soluble in cold water but more soluble in hot water, and cystine is sparingly soluble in both hot and cold water. They are all soluble in dilute acids and alkalies with the exception of cystine, which dissolves with difficulty in dilute ammonia water. All the amino acids possess at least one free amino group (NH_2) and one free carboxyl group (COOH), except proline and oxyproline, which contain an imino (NH) group instead of an amino group. This means that they are amphoteric compounds, being capable of reacting with both acids and bases (page 174). Most of the acids have one free amino group and one free carboxyl group. These are known as mono-amino-mono-carboxylic acids and are neutral in reaction. Some have two amino groups to one carboxyl group. These are di-amino-mono-

carboxylic acids and are alkaline in reaction. Others have one amino to two carboxyl groups. These are mono-amino-di-carboxylic acids and are acid in reaction.

The amino acids are not secondary products of protein decomposition. They preexist in the protein molecule. Many theories have been advanced to explain how the acids are linked together to produce a protein molecule. It is generally agreed that the amino acids in proteins are linked together principally through their α-amino and carboxyl groups to produce the so-called peptid linkage (R·CO—NH·R[1]). Other types of linkage probably occur but, from the known facts, the peptid linkage is the principal one. For example, if a tripeptid is composed of glycine, alanine, and serine, the amino acids would be joined together in the following manner, according to the peptid linkage:



Glycyl-alanyl-serine

The hydrolysis of proteins by proteolytic enzymes results in the formation of the following fractions and in the order named:

Proteins → proteoses (albumoses) → peptones → peptids → amino acids

Enzymes that open up or hydrolyze peptid linkages are grouped under the proteases. The proteases are further subdivided into the proteinases and peptidases. The proteinases attack the true proteins. They do not hydrolyze proteins beyond the polypeptid or dipeptid stage. The peptidases hydrolyze polypeptids and dipeptids to the stage of amino acids. This may be schematically represented as shown in Fig. 131.

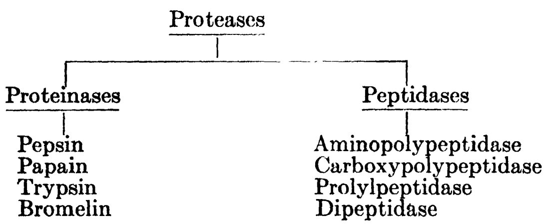


Fig. 131.—Classification of some of the proteases.

Berger, Johnson, and Peterson (1938*a,b*) investigated the peptidases elaborated by the following organisms: *Bacillus megatherium, B. mesentericus, B. subtilis, Clostridium acetobutylicum, C. sporogenes, Escherichia coli, Lactobacillus plantarum, Leuconostoc mesenteroides, Granulobacter butylicum, Phytomonas tumefaciens, Propionibacterium pentosaceum, Proteus vulgaris,* and *Pseudomonas fluorescens.* The specificity of the

peptidase systems of *B. megatherium* and *E. coli* was studied in some detail. Dipeptids and tripeptids were readily hydrolyzed but acylated or decarboxylated peptids were hydrolyzed only very slowly or not at all Substitution of a methyl group for a hydrogen atom of the free amino group on a peptid resulted in a very marked decrease in hydrolysis. The peptidase system from *L. mesenteroides* is capable of hydrolyzing both optical components of the racemic peptids leucylglycine, leucyldiglycine, alanylglycine, and alanyldiglycine. The peptidase complex appears to contain at least two dipeptid-splitting enzymes, at least three polypeptidases, and an acylase but no carboxypolypeptidase. Appreciable amounts of peptidases were found in culture filtrates of *E. coli* and *B. megatherium.* With *E. coli* more enzymes could be extracted from the cells than from the medium on which they were grown. This is to be expected with a nonproteolytic organism. With the proteolytic organism *B. megatherium,* more peptidases were consistently found in a given volume of medium than could be obtained from the cells grown on the same volume of medium.

For more information on proteolytic enzymes see the report by Bergmann and Fruton (1941).

Protein Decomposition.—Protein decomposition is defined in various ways but the term is used here to indicate the action of proteolytic enzymes on a protein, resulting in the disruption of the colloidal molecule into diffusible substances. The bacterial enzymes responsible for the action are probably all extracellular and hydrolytic in nature. As has already been explained (page 262), the function of the extracellular enzymes is to convert insoluble, indiffusible compounds into soluble, diffusible substances which can be absorbed by the bacterial cell. The compounds produced are probably without odor. The extracellular enzymes play no part in the respiratory activities of the cell, such function being performed by the intracellular enzymes.

Putrefaction.—The term putrefaction may be defined as the anaerobic decomposition of proteins, protein split products, and nitrogenous compounds of a similar nature, with the formation of foul-smelling compounds. Probably all the reactions take place inside the cell by means of the intracellular or respiratory enzymes. The putrefactive changes are the result of the action of organisms on the individual amino acids. The compounds produced include methyl mercaptan, hydrogen sulfide, amines, alcohols, organic acids, hydroxy acids, indole, phenol, cresol, ammonia, methane, carbon dioxide, hydrogen. etc.

The putrefactive anaerobes include *Clostridium aerofoetidum, C. bifermentans, C. flabelliferum, C. histolyticum, C. lentoputrescens, C. ovalaris, C. parabotulinum, C. parasporogenes, C. putrefaciens, C. sporogenes, C. tyrosinogenes,* etc.

Bacteria vary considerably in their ability to degrade proteins or protein split products. Organisms are usually designated as putrefactive or fermentative, depending upon whether they act more vigorously on proteins or on fermentable substances. Many of the putrefactive compounds are produced only from specific amino acids whereas others may be produced from more than one acid.

Decay.—Decay may be defined as the aerobic decomposition of proteins in which the products of putrefaction are completely oxidized to stable compounds having no foul odors. This action takes place by aerobic organisms in the presence of a plentiful supply of air after the putrefactive changes have occurred. If the substrate is well aerated from the start, the changes will be aerobic without the formation of foul-smelling compounds.

A practical application of this principle is employed in the disposal of sewage. In one process the sewage is first digested by the anaerobic organisms resulting in the liberation of offensive odors. The anaerobic digestate is then well aerated, after which the aerobic organisms digest the foul-smelling compounds, resulting in the disappearance of the offensive odors. In another process the sewage is kept well aerated from the start, thus preventing the growth of anaerobic organisms. The aerobic digestion occurs without the formation of ill-smelling compounds.

ACTION OF BACTERIA ON PROTEINS

Bainbridge (1911) first reported that native proteins are resistant to bacterial attack even by the most proteolytic species. He employed an inorganic medium to which was added egg albumin and other proteins as the only source of nitrogen and carbon. The test organisms included *E. coli, Eberthella typhosa, Proteus vulgaris, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella enteritidis,* and *Neisseria gonorrhoeae.* The organisms not only failed to degrade the pure proteins but showed no increase in numbers. If, however, a small amount of peptone was added to the medium, multiplication of the organisms and degradation of the proteins took place.

Later Sperry and Rettger (1915) confirmed and extended the observations of Bainbridge. They employed aerobic, anaerobic, and facultative species in both nonsynthetic and synthetic media. None of the species employed was capable of degrading the proteins. The addition of a trace of peptone resulted in the decomposition of the proteins.

The results appear to indicate that an extracellular enzyme is necessary to convert the indiffusible proteins into diffusible compounds. In the absence of an available nitrogen and carbon source the organisms are unable to multiply and elaborate the necessary enzyme or enzymes. The addition of peptone furnishes a utilizable source of nitrogen and

carbon thus permitting the organisms to multiply and elaborate the necessary extracellular proteolytic enzymes.

Berman and Rettger (1916, 1918) showed that proteoses are also immune to attack by bacteria. Proteoses or albumoses are intermediate products in the d gestion of proteins by proteolytic enzymes. Here again the addition of a small amount of peptone to the medium stimulated the organisms to secrete the proteolytic enzyme or enzymes necessary to attack the proteoses.

The results indicate that, in the case of the nonproteolytic bacteria at least, proteins and higher split fractions such as the proteoses are not available as sources of nitrogen and carbon. Peptids and amino acids appear to be essential nitrogenous food constituents. This explains why commercial peptones are so extensively employed in bacteriological culture media.

ACTION OF BACTERIA ON AMINO ACIDS

Amino acids may be broken down in a variety of ways. The types of chemical reactions involved include the following:

1. Hydrolytic deaminization resulting in the formation of a hydroxy acid:

$$\underset{\text{Amino acid}}{\begin{array}{c}R\\ |\\ H-C-NH_2\\ |\\ COOH\end{array}} + H_2O \rightarrow \underset{\text{Hydroxy acid}}{\begin{array}{c}R\\ |\\ H-C-OH\\ |\\ COOH\end{array}} + NH_3$$

2. Decarboxylation or elimination of CO_2 resulting in the formation of an amine with one less carbon atom:

$$\begin{array}{c}R\\ |\\ H-C-NH_2\\ |\\ COOH\end{array} \rightarrow \underset{\text{Amine}}{\begin{array}{c}R\\ |\\ H-C-NH_2\\ |\\ H\end{array}} + CO_2$$

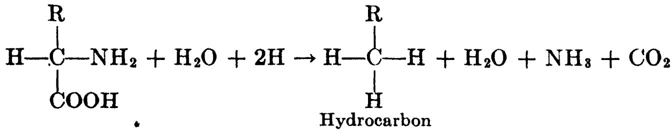
3. Deaminization and decarboxylation resulting in the formation of a primary alcohol with one less carbon atom:

$$\begin{array}{c}R\\ |\\ H-C-NH_2\\ |\\ COOH\end{array} + H_2O \rightarrow \underset{\text{Primary alcohol}}{\begin{array}{c}R\\ |\\ H-C-OH\\ |\\ H\end{array}} + CO_2 + NH_3$$

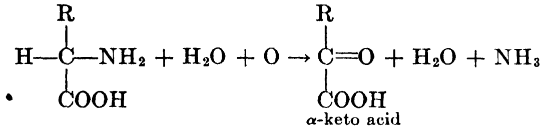
4. Deaminization and reduction resulting in the formation of a saturated acid:

$$\begin{array}{c}R\\ |\\ H-C-NH_2\\ |\\ COOH\end{array} + H_2O + 2H \rightarrow \underset{\text{Saturated acid}}{\begin{array}{c}R\\ |\\ H-C-H\\ |\\ COOH\end{array}} + H_2O + NH_3$$

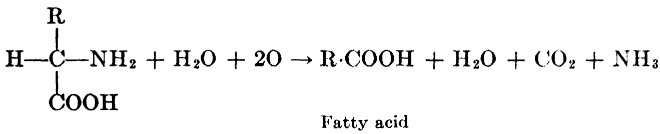
5. Deaminization, decarboxylation, and reduction resulting in the formation of a hydrocarbon:



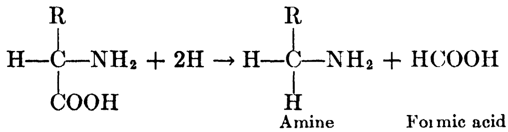
6. Deaminization and oxidation resulting in the formation of an α-keto acid:



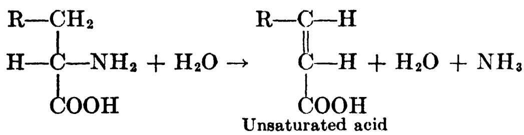
7. Deaminization, decarboxylation, and oxidation resulting in the formation of a fatty acid with fewer C atoms:



8. Reduction and elimination of formic acid resulting in the formation of an amine with one less C atom:



9. Deaminization and desaturation at the α-β-linkage resulting in the formation of an unsaturated acid:



10. Anaerobic decomposition resulting in the liberation of hydrogen.

The foregoing types of chemical reactions or their combinations are able to account for all or nearly all the products of decomposition and putrefaction produced by bacteria from amino acids.

In Table 39 are included the names and formulas of the amino acids present in proteins and the products produced by bacterial action. The list does not include all possible compounds but only those which have been recorded as being produced by bacteria. The numbers refer to the types of chemical reactions already given.

TABLE 39.—ACTION OF BACTERIA ON THE AMINO ACIDS

Amino acid	Compounds produced
Glycine or glycocoll, $CH_2NH_2\cdot COOH$	(2) Methylamine, CH_3NH_2 (4) Acetic acid, $CH_3\cdot COOH$ (5) Methane, CH_4
Alanine, $CH_3\cdot CHNH_2\cdot COOH$	(2) Ethylamine, $C_2H_5NH_2$ (3) Ethyl alcohol, C_2H_5OH (4) Propionic acid, $C_2H_5\cdot COOH$ (7) Acetic acid, $CH_3\cdot COOH$
Valine, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\cdot CHNH_2\cdot COOH$	(2) Isobutylamine, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\ CH_2NH_2$ (4) Isovaleric acid, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\ CH_2\cdot COOH$ (7) Isobutyric acid, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\cdot COOH$ and Acetic acid, $CH_3\cdot COOH$ Formic acid, $HCOOH$
Leucine, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\cdot CH_2\cdot CHNH_2\cdot COOH$	(1) Leucic acid (isopropyl lactic acid), $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\cdot CH_2\cdot CHOH\cdot COOH$ (2) Isoamylamine, $\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CH\cdot CH_2\cdot CH_2NH_2$
Isoleucine, $\begin{matrix}CH_3\\C_2H_5\end{matrix}\!\!>\!CH\cdot CHNH_2\cdot COOH$	(4) Methylethylpropionic acid (*d*-caproic acid), $\begin{matrix}CH_3\\C_2H_5\end{matrix}\!\!>\!CH\cdot CH_2\cdot COOH$ (7) Methylethylacetic acid, $\begin{matrix}CH_3\\C_2H_5\end{matrix}\!\!>\!CH\cdot COOH$

TABLE 39.—(*Continued*)

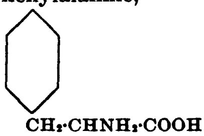
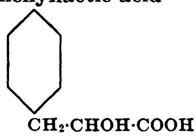
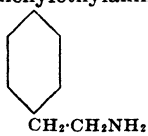
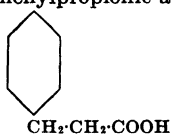
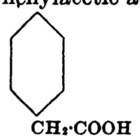
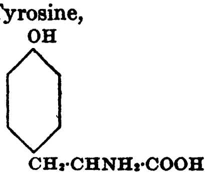
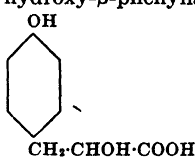
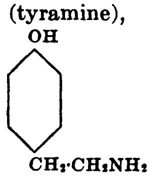
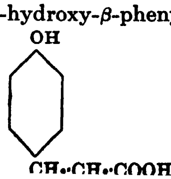
Amino acid	Compounds produced
Serine, CH_2OH | $CHNH_2$ | $COOH$	(2) Aminoethyl alcohol, $CH_2OH{\cdot}CH_2NH_2$ (3) Ethylene glycol, $CH_2OH{\cdot}CH_2OH$ (4) Propionic acid, $C_2H_5{\cdot}COOH$ (7) Formic acid, HCOOH
Phenylalanine, 	(1) Phenyllactic acid  (2) Phenylethylamine,  (4) Phenylpropionic acid,  (7) Phenylacetic acid, 
Tyrosine, 	(1) *p*-hydroxy-β-phenyllactic acid,  (2) *p*-hydroxy-β-phenylethylamine (tyramine),  (4) *p*-hydroxy-β-phenylpropionic acid, 

TABLE 39.—(*Continued*)

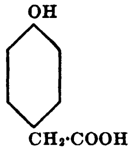
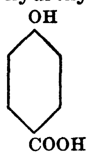
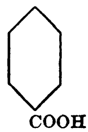
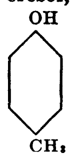
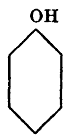
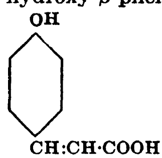
Amino acid	Compounds produced
	(7) *p*-hydroxyphenylacetic acid,  (7) *p*-hydroxybenzoic acid,  (7) Benzoic acid,  (7) *p*-cresol,  (7) Phenol (carbolic acid),  (9) *p*-hydroxy-β-phenylacrylic acid 
Cystine, COOH COOH | | CHNH$_2$ CHNH$_2$ | | CH$_2$—S—S—CH$_2$	Cysteine, COOH | CHNH$_2$ | CH$_2$SH Thioglycollic acid, COOH·CH$_2$SH Methyl mercaptan, CH$_3$SH Hydrogen sulfide, H_2S Acetic acid, CH$_3$·COOH Formic acid, HCOOH Hydrogen and carbon dioxide, H_2 and CO_2

TABLE 39.—(*Continued*)

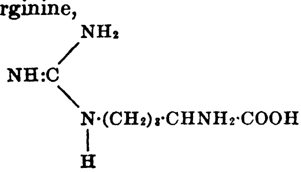
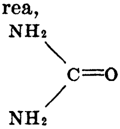
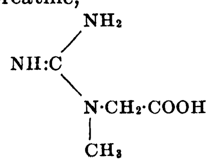
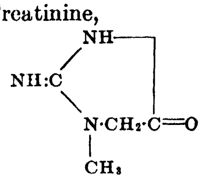
Amino acid	Compounds produced
Aspartic acid, COOH CH_2 $CHNH_2$ COOH	(1) Malic acid, $COOH{\cdot}CH_2{\cdot}CHOH{\cdot}COOH$ (2) β-alanine, $COOH{\cdot}CH_2{\cdot}CH_2NH_2$ (4) Succinic acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ (5) Propionic acid $CH_3{\cdot}CH_2{\cdot}COOH$ (7) Acetic acid, $CH_3{\cdot}COOH$ and formic acid, HCOOH (9) Fumaric acid, $COOH{\cdot}CH{:}CH{\cdot}COOH$ (10) Hydrogen and carbon dioxide, H_2 and CO_2
Glutamic acid, COOH CH_2 CH_2 $CHNH_2$ COOH	(2) γ-aminobutyric acid (piperidinic acid), $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2NH_2$ (4) Glutaric acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ (5) Butyric acid, $CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ and formic acid, HCOOH (7) Succinic acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$ (7) Acetic acid, $CH_3{\cdot}COOH$ (7) Formic acid, HCOOH (10) Succinic acid, $COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH$, Acetic acid, $CH_3{\cdot}COOH$, Hydrogen and carbon dioxide, H_2 and CO_2
Arginine, 	Ornithine, $NH_2CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH$ and Urea,  Creatine,  Creatinine,  Further decomposition of arginine is the result of the action of organisms on ornithine

TABLE 39.—(*Continued*)

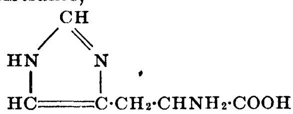
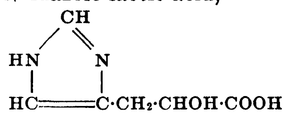
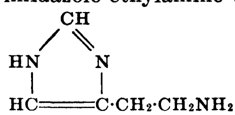
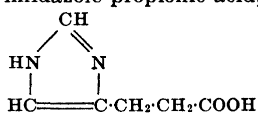
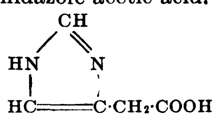
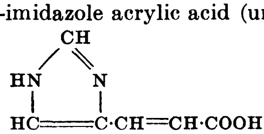
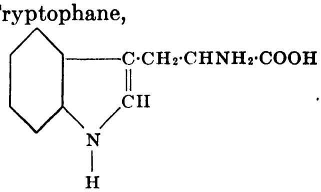
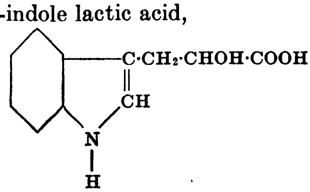
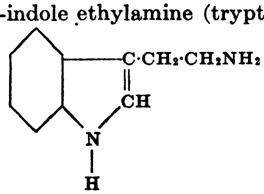
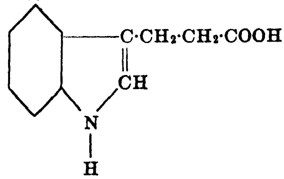
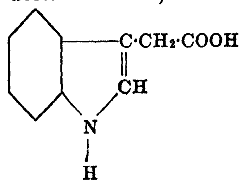
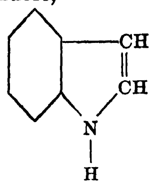
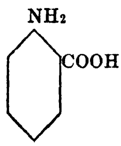
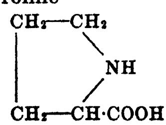
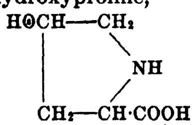
Amino acid	Compounds produced
Ornithine, $NH_2CH_2 \cdot CH_2 \cdot CH_2 \cdot CHNH_2 \cdot COOH$	(2) Tetramethylenediamine (putrescine), $NH_2CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2NH_2$ (4) *d*-aminovaleric acid (putridin), $NH_2CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$
Lysine, $NH_2CH_2 \cdot (CH_2)_3 \cdot CHNH_2 \cdot COOH$	(2) Pentamethylenediamine (cadaverine), $NH_2CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2NH_2$ (4) ϵ-aminocaproic acid, $NH_2CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$
Histidine, 	(1) β-imidazole lactic acid,  (2) β-imidazole ethylamine (histamine),  (4) β-imidazole propionic acid,  (7) Imidazole acetic acid.  (9) β-imidazole acrylic acid (urocanic acid),  (10) Hydrogen and carbon dioxide
Tryptophane, 	(1) β-indole lactic acid,  (2) β-indole ethylamine (tryptamine), 

TABLE 39.—(*Continued*)

Amino acid	Compounds produced
	(4) β-indolepropionic acid (skatoleacetic acid),  (7) Indoleacetic acid,  Indole,  Anthranilic acid, 
Proline 	δ-aminovaleric acid, $NH_2CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$ Valeric acid, $CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$
Hydroxyproline, 	No reactions reported
Methionine, $CH_3 \cdot S \cdot (CH_2)_2 \cdot CHNH_2 \cdot COOH$	No reactions reported

For an excellent review of the action of bacteria on amino acids see the monograph by Gale (1940).

PTOMAINES

Ptomaines may be defined as basic amines and diamines, which are formed by the action of putrefactive bacteria on proteins, amino acids, and organic bases. They are produced from amino acids by a process of decarboxylation.

The ptomaines formed by the decarboxylation of some of the amino acids are given in Table 40. The organic base choline is included as an example of a compound that is not an amino acid.

PROTEIN-SPARING ACTION

Kendall and Walker (1915) found that the presence of a deficient amount of glucose in a gelatin medium inhibited the secretion of a gelatinolytic enzyme until all of the carbohydrate was utilized. In the presence of an excess of glucose, however, the enzyme never appeared. Control experiments ruled out the possibility that glucose inhibited the action of the enzyme subsequent to its secretion. The question of whether the enzyme was inhibited in its action by the presence of

TABLE 40.—SOME PTOMAINES FORMED BY BACTERIAL ACTION

Amino Acid	Ptomaine
Glycine (glycocoll)	Methylamine
Alanine	Ethylamine
Valine	Isobutylamine
Leucine	Isoamylamine
Phenylalanine	Phenylethylamine
Tyrosine	p-hydroxy-β-phenylethylamine (tyramine)
Serine	Hydroxyethylamine
Cystine (cysteine)	Thioethylamine
Arginine (ornithine)	Tetramethylenediamine (putrescine)
Lysine	Pentamethylenediamine (cadaverine)
Histidine	β-imidazole ethylamine (histamine)
Tryptophane	β-indole ethylamine (tryptamine)
Choline	Trimethylamine

the high acidity was also ruled out since the enzyme appeared after all of the carbohydrate was fermented. They concluded that organisms preferred a fermentable carbohydrate to protein for energy purposes. The nitrogenous constituents were utilized for structure only. In the absence of a fermentable carbohydrate the bacteria were forced to utilize the protein constituents for both structure and energy.

Berman and Rettger (1918) and others criticized the conclusions of Kendall and his school. They found that organisms like *B. subtilis*, which ferment glucose very slowly, or those like *Aerobacter cloacae*,

which do not produce strongly acid products, failed to show a protein-sparing action. Protein breakdown occurred as though there was no carbohydrate present. On the other hand, organisms such as *E. coli* and *Proteus vulgaris*, which produced considerable amounts of acid in a short period of time, were inhibited in growth and chemical activity after a brief incubation period. Organisms in this group showed a definite protein-sparing action. In the presence of an excess of buffer, such as a mixture of primary and secondary phosphates, the limiting hydrogen-ion concentration was never reached and the course of protein breakdown occurred as rapidly in the presence of a fermentable carbohydrate as in its absence. The result was a negative, protein-sparing action.

It may be concluded that the presence of a fermentable carbohydrate exerts a protein-sparing action only when it is rapidly utilized with the accumulation of a relatively large amount of acid in a short period of time. The high acidity inhibits growth of the organisms at an early stage before they have had sufficient time to elaborate proteolytic enzymes.

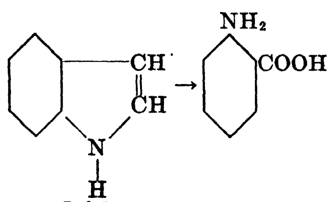
SOME ROUTINE PHYSIOLOGICAL REACTIONS EMPLOYED FOR THE IDENTIFICATION OF BACTERIA

Many biochemical reactions are employed for the identification and classification of bacteria. Some of the reactions are based on the breakdown of carbohydrates; others depend upon certain changes in the nitrogenous constituents of the medium.

Production of Indole.—Indole is a putrefactive compound produced by the action of some bacteria on the amino acid tryptophane (page 317). Since no other amino acid contains the indole ring, the test is specific for the presence of tryptophane.

Tryptophane is not present in all proteins. Manufacturers select only those proteins for peptone production which are certain to yield a relatively high content of tryptophane. Casein, the principal protein of milk, contains a large amount of this acid and is often used for the preparation of peptone where a strong indole test is required.

There is evidence to show that some organisms are capable of oxidizing the indole ring. Supniewski (1924) reported that *Pseudomonas aeruginosa* oxidized indole to anthranilic acid:



Indole Anthranilic acid

Sasaki (1923) showed that *B. subtilis* not only produced indole from tryptophane but also oxidized it to anthranilic acid. Gray (1928) isolated an organism from soil, *P. indoloxidans*, which oxidized indole to indigotin (indigo blue). Crystals of the compound appeared around colonies of the organisms on agar plates. Surprisingly, this organism is unable to oxidize tryptophane to indole. Kotake (1933) showed that *B. subtilis* was capable of producing kynurenic acid and anthranilic acid from tryptophane in the presence of glycerol and aluminum phosphate.

In order to prevent the possible occurrence of a protein-sparing action, noncarbohydrate media should be employed for the detection of indole in bacterial cultures. The test is of value in identifying and classifying bacteria.

Production of Ammonia.—The development of ammonia in bacterial cultures results largely from the deaminization of the amino acids present in culture media.

It is generally believed that amino acids must be first deaminized prior to assimilation. In the absence of a fermentable carbohydrate, organisms utilize the ammonia for structure and the deaminized carbon chains for energy. Since more carbon is required for energy than nitrogen for structure, ammonia will accumulate in the medium. In the presence of a fermentable carbohydrate a protein-sparing action may occur, in which case the organisms utilize the amino acids for structure but not for energy. A carbohydrate medium inoculated with a vigorous fermenter usually shows less free ammonia than the same medium without carbohydrate. This may be attributed to two factors: (1) Organisms utilize carbohydrate in preference to the deaminized amino acids for energy. Since the carbon of the amino acids is not required for energy, less ammonia is produced. (2) The addition of a fermentable carbohydrate to a medium stimulates bacterial growth (increase in numbers), resulting in an increased utilization of ammonia. This causes a decrease in the free ammonia content of the medium. It is generally believed that both factors apply. This means that in the case of the strongly fermentative organisms the addition of a carbohydrate to a culture results in the accumulation of less free ammonia than in the same culture medium not containing carbohydrate.

Peptonization and Fermentation of Milk.—According to Van Slyke and Bosworth (1915) the constituents of milk may be placed in three groups on the basis of their solubilities. Their classification, with additions by Hawk and Bergeim (1937), is as follows:

I. Milk constituents in true solution:

1. Lactose.
2. Citric acid.
3. Potassium.
4. Sodium.
5. Chlorine.
6. Vitamin B_1
7. Vitamin G (riboflavin)
8. Vitamin C.

II. Milk constituents partly in solution and partly in suspension or colloidal solution:
1. Albumin.
2. Inorganic phosphate.
3. Calcium.
4. Magnesium.

III. Milk constituents entirely in suspension or colloidal solution:
1. Fat.
2. Casein.
3. Vitamin A.
4. Vitamin D.

The use of milk as a culture medium dates back to the beginning of bacteriology. It is used as a differential medium to demonstrate the ability of an organism to produce a fermentation, or a peptonization, or a simultaneous fermentation and peptonization.

Casein is a protein capable of reacting both as a weak acid and as a weak base. It is present in milk entirely in colloidal suspension. Some bacteria secrete a rennin-like enzyme capable of hydrolyzing casein to soluble *p*-casein and a compound similar to peptone. The soluble *p*-casein then reacts with the calcium salts in solution, resulting in the formation of a precipitate of *p*-casein or calcium *p*-caseinate. The clear liquid surrounding the curd of *p*-casein is known as whey. This may be schematically represented as follows:

Casein + rennin

Peptone-like body | Soluble *p*-casein

+ Calcium salts

p-casein
(Calcium *p*-caseinate)

Bacteria capable of vigorously attacking the lactose produce a high degree of acidity resulting in the precipitation or curdling of the casein. The clear supernatant fluid is also known as whey. The final pH is sufficient to prevent further bacterial action. Since carbohydrate spares protein, no putrefactive changes occur (Fig. 132).

A rennin curd is usually followed by a peptonization of the casein. This means that the organisms either do not attack the lactose or ferment it very slowly. In the former instance the casein is first hydrolyzed and then decomposed, with the formation of various soluble products. In the latter case a slow action on the milk sugar results in a simultaneous fermentation and protein decomposition, owing to the fact that the lactose exhibits no protein-sparing action.

The indicator litmus is frequently added to milk to detect acid production and also the ability of an organism to decolorize it to its colorless form. The newer, more brilliant indicators such as bromocresol purple are superior to litmus for detecting acid production, owing to their greater sensitivity, but they do not exhibit reduction phenomena that are often of diagnostic importance.

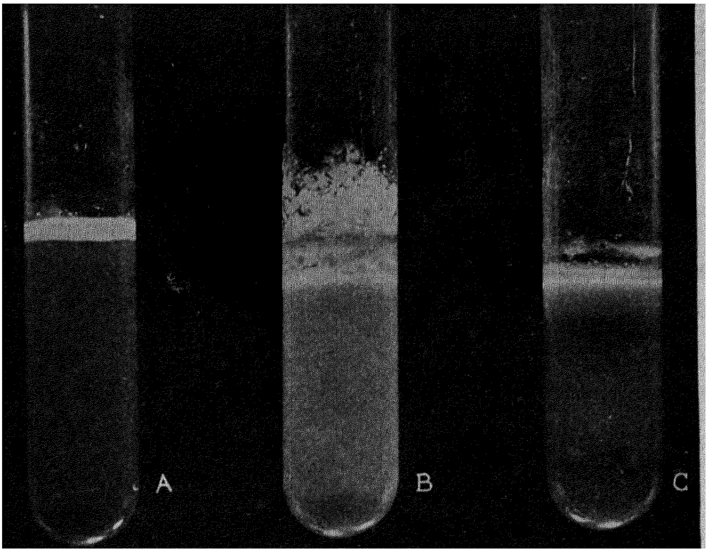


FIG. 132.—Fermentation and peptonization of milk. *A*, litmus milk uninoculated; *B* inoculated with *Escherichia coli*, showing acid curd with decolorization of the indicator; *C*, inoculated with *Bacillus subtilis*, showing peptonization.

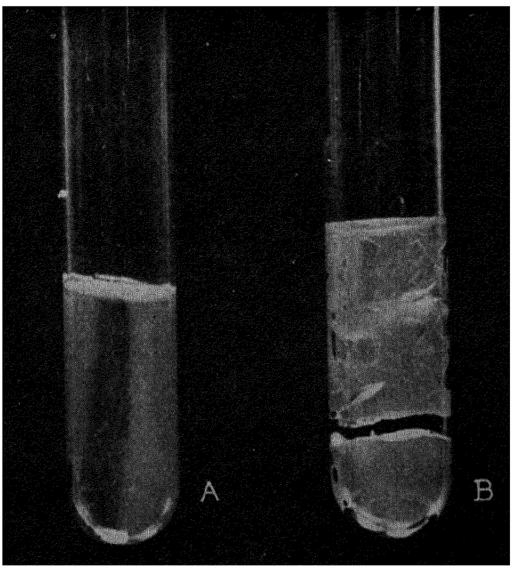


FIG. 133.—Production of hydrogen sulfide. *A*, *Proteus vulgaris*, the blackening of the agar along the line of stab is due to the formation of hydrogen sulfide; *B*, *Escherichia coli*, this organism does not produce hydrogen sulfide in sufficient quantity to give a positive test. The separation of the agar is due to the fermentation of the glucose in the medium to acid and gas.

Production of Hydrogen Sulfide.—Cystine and methionine are the two sulfur-containing amino acids found in proteins. Cystine is probably of greater occurrence and is, therefore, more important from a bacteriological standpoint. Cystine does not occur in all proteins. Therefore only those proteins are selected for peptone production which are certain to contain relatively large amounts of cystine.

Some organisms are capable of dissimilating cystine with the production of hydrogen sulfide as one of the putrefactive products. It is usually stated that the first change that occurs is a reduction of one molecule of cystine to two molecules of cysteine (page 315). Tarr (1933) believed that cystine undergoes an anaerobic reduction to give hydrogen sulfide and other products.

Hydrogen sulfide reacts with heavy metals to produce colored compounds. The metal salts are incorporated in solid media. The presence of hydrogen sulfide is detected by the darkening of the medium along the line of inoculation (Fig. 133). The metals commonly employed are lead, iron, and bismuth. Tittsler and Sandholzer (1937) found iron to be more sensitive than lead. Recently Hunter and Crecelius (1938) reported that bismuth appeared to be more sensitive than either lead or iron for the detection of hydrogen sulfide in cultures. Organisms that produce a small amount of hydrogen sulfide gave negative results with iron and lead, and definite browning occurred in bismuth sulfite medium. More recently Utermohlen and Georgi (1940) employed a medium containing a mixture of cobalt and nickel salts and found it to be superior to both iron and lead and probably as good as bismuth for hydrogen sulfide detection. It may be concluded that it is not enough to state merely than an organism does or does not produce hydrogen sulfide without stating the conditions under which it was investigated.

Organisms can be placed in two groups on the basis of hydrogen sulfide production. The test is of value in identifying and classifying bacteria.

Liquefaction of Gelatin.—The liquefaction of gelatin is due to the presence of an enzyme known as gelatinase. It is an extracellular enzyme, which is concerned with the hydrolysis of the protein prior to diffusion and intracellular utilization.

The presence of the enzyme may be demonstrated by inoculating a tube of gelatin with the organism to be examined and incubating at the proper temperature. If the temperature of incubation is above 20°C., the gelatin will melt. Under these conditions the presence of gelatinase may be determined by placing the gelatin in the refrigerator and noting whether or not it hardens. If the gelatin remains liquid, it shows that the organism under examination secreted a gelatinase into the culture medium.

The extracellular nature of the enzyme may be demonstrated by filtering a culture of an appropriate organism and adding some of the filtrate to a tube of gelatin medium. The presence of a gelatinase will result in a liquefaction of the gelatin.

Another method used for demonstrating the presence of the enzyme in a culture is to add just sufficient phenol to kill the organisms but not enough to have any appreciable effect on the enzyme. The addition of some of this phenolated culture to a tube of gelatin will produce a liquefaction if the organism under examination is capable of elaborating a gelatinase.

A protein-sparing action results in a negative test for gelatin liquefaction. Therefore, noncarbohydrate media should be employed for demonstrating the ability of an organism to secrete a gelatinase. The test is of value in identifying and classifying bacteria.

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CHAPTER XV

FERMENTATION OF CARBOHYDRATES AND RELATED COMPOUNDS

The term fermentation has undergone many changes in meaning since the time of Pasteur but it is defined here as the incomplete oxidation of carbohydrates and carbohydrate-like compounds by microorganisms. The various types of fermentations result in the formation of many kinds of organic acids and other compounds. Some of these compounds are formic, acetic, propionic, butyric, valeric, succinic, lactic, citric, oxalic, fumaric, kojic, and gluconic acids, acetone, glycerol, ethyl alcohol, butyl alcohol, isopropyl alcohol, and the gases methane, hydrogen, and carbon dioxide. This is only a partial list but includes probably the most important compounds elaborated by organisms in the various types of fermentations.

Bacterial action on carbohydrates may be either aerobic or anaerobic. In the presence of sufficient oxygen, carbohydrate breakdown may proceed to the final end products carbon dioxide and water. In the absence of free oxygen incomplete combustion and anaerobic breakdown occur. In anaerobic breakdown organisms are unable to obtain the maximum amount of energy available in fermentable compounds.

If glucose is taken as an example of a fermentable compound that is utilized for purposes of energy, the (1) complete oxidation, (2) partial oxidation, and (3) anaerobic decomposition yield the following number of calories:

1. Complete oxidation:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 674 \text{ calories}$$

2. Partial oxidation:

$$C_6H_{12}O_6 + 4\tfrac{1}{2}O_2 \rightarrow \underset{\text{Oxalic acid}}{3(COOH)_2} + 3H_2O + 493 \text{ calories}$$

3. Anaerobic decomposition:

(a) $$C_6H_{12}O_6 \rightarrow \underset{\text{Lactic acid}}{2CH_3{\cdot}CHOH{\cdot}COOH} + 22\tfrac{1}{2} \text{ calories}$$

(b) $$C_6H_{12}O_6 \rightarrow \underset{\text{Ethyl alcohol}}{2CH_3{\cdot}CH_2OH} + 2CO_2 + 22 \text{ calories}$$

(c) $$C_6H_{12}O_6 \rightarrow \underset{\text{Acetic acid}}{3CH_3{\cdot}COOH} + 15 \text{ calories}$$

The fermentation of glucose by *Escherichia coli* yields formic, acetic, lactic, and succinic acids, ethyl alcohol, hydrogen, and carbon dioxide. Grey (1919) showed that 100 gm. of the above products on complete oxidation yielded about 316 cal. but only about 58 cal. were liberated in the reaction. The incomplete oxidation of glucose by *E. coli* yielded only about 16 per cent of the potential energy.

RETTING OF FLAX AND HEMP

Celluloses, hemicelluloses, and pectins are polysaccharides, which together form the framework of plants. The hemicelluloses differ from the celluloses in being more easily hydrolyzed with dilute mineral acids and in giving different products when hydrolyzed. The hemicelluloses include

The hexosans:
1. The galactans yield *d*-galactose on hydrolysis.
2. The mannans yield principally *d*-mannose and a small amount of *d*-fructose on hydrolysis.

The pentosans:
1. The xylans yield *l*-xylose on hydrolysis.
2. The arabans yield *l*-arabinose on hydrolysis.

Hemicelluloses are attacked by bacteria with difficulty but are easily hydrolyzed by molds.

Pectins accompany celluloses and hemicelluloses in making up the framework of plants. They are compounds of a gelatinous consistence which form the middle lamellar layer of plant cells. Pectins act as cement-like substances that bind the cells together. Pectins from different sources appear to have the same general chemical composition, being calcium and magnesium salts of a complex carbohydrate association. Hydrolysis of pectins yields chiefly galacturonic acid, *l*-arabinose, and *d*-galactose, accompanied by small amounts of methyl alcohol, acetic acid, calcium, magnesium, etc.

The bacterial hydrolysis of pectin is of great importance in the retting or rotting of flax and hemp. This may be accomplished by both anaerobic and aerobic methods.

Anaerobic Retting.—Retting is carried out by immersing the flax or hemp stalks in water and weighting them down. Water is absorbed by the tissues, causing swelling and the extraction of water-soluble substances. The substances that are extracted amount to about 12 per cent of the weight of the plants and consist of sugars, glucosides, tannins, soluble nitrogenous compounds, and coloring matter. The highly colored water now becomes a good culture medium for the growth of many organisms. The aerobic organisms grow first, utilizing the dissolved oxygen and in so doing create an environment compatible to the growth

requirements of the anaerobes. The pectin is slowly fermented and dissolved by the anaerobes, leaving the fibers intact. During the fermentation various organic acids and gases are produced. These include chiefly acetic and butyric acids, carbon dioxide, and hydrogen. About 10 days are required for the reaction to go to completion. The flax or hemp should be removed from the water when the reaction has gone to completion, otherwise overretting will result. The bundles are thoroughly washed to remove the organic acids, odors, and other undesirable substances, and then spread out in the sun or air to dry. The dried material is then ready for scutching.

The organism responsible for the hydrolysis and fermentation of pectin is known as *Clostridium butyricum*. It is a motile, anaerobic, spore-bearing, Gram-positive rod. The organism ferments a number of carbohydrates and carbohydrate-like compounds, including xylose, glucose, sucrose, starch, salicin, and mannitol, with the production of acid and gas. The fermentable products include butyl, ethyl, and isopropyl alcohols, acetone, organic acids, hydrogen, and carbon dioxide. The rods are capable of utilizing atmospheric nitrogen. The organism grows best at a temperature of 30 to 37°C.

Recent improvements on the process make use of the anaerobic organism *C. felsineum*. A culture of the organism is prepared and added to the water of the retting vat in the proportion of 1000 cc. to 10,000 gm. of dry tissue. The vat is kept at a temperature of 37 to 38°C. for a period of from 50 to 75 hr. The process requires closer supervision than the preceding method.

Aerobic Retting.—An aerobic process known as dew retting is also employed. The stalks are spread on the ground in the fall and allowed to remain throughout the winter months. A disadvantage to the method is that it is too slow, months instead of days being required. The organism chiefly responsible for the reaction is the mold *Mucor stolonifer*. Other organisms, both molds and bacteria, are doubtless concerned in the reaction.

ALCOHOLIC FERMENTATION

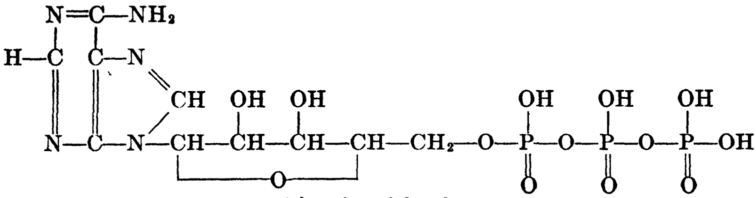
Alcohol is produced commercially by means of yeasts. The species of yeast that is generally used for this purpose is *Saccharomyces cerevisiae*, the ordinary baker's or brewer's yeast. The organism converts approximately 90 per cent of the sugar into equimolecular quantities of alcohol and carbon dioxide. The final equation may be written:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow 2CO_2 + \underset{\text{Ethyl alcohol}}{2C_2H_5OH}$$

Many theories have been proposed to explain the mechanism of alcoholic fermentation. The theory generally believed to explain the

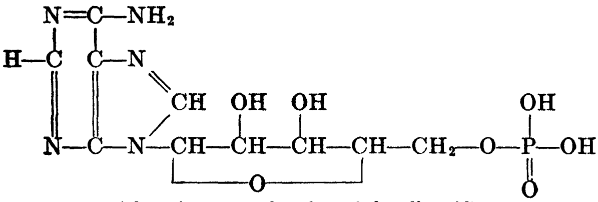
facts better than any of the others is based on the work of Embden, Meyerhof, and Parnas.

The first step in the reaction involves the phosphorylation of the hexose by the coenzyme adenosine triphosphate.



Adenosine triphosphate

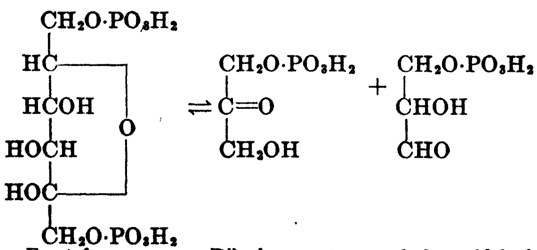
The coenzyme is present in yeast juice and is capable of transferring phosphate to the hexose, converting the sugar first to hexose monophosphate and then to hexosediphosphate. The loss of one molecule of phosphate converts the coenzyme to adenosine diphosphate; the loss of two molecules of phosphate converts it to adenosine monophosphate or adenylic acid.



Adenosine monophosphate (adenylic acid)

The phosphate is not utilized in the reaction. When the fermentation reaches the pyruvic acid stage, the phosphate is liberated and becomes available again for phosphorylating additional sugar.

The phosphorylation of glucose, fructose, or mannose, in the presence of the coenzyme adenosine triphosphate, yields the same compound, namely, fructofuranose 1:6-diphosphate. This compound splits to form one molecule of dihydroxyacetone phosphate and one molecule of 3-glyceraldehyde phosphate which are in equilibrium with the hexose diphosphate.



Fructofuranose 1 : 6-diphosphate — Dihydroxyacetone phosphate — 3-glyceraldehyde phosphate

Dihydroxyacetone phosphate and 3-glyceraldehyde phosphate are also in equilibrium with each other, being converted largely into the former compound.

$$\begin{array}{ccc} CH_2O{\cdot}PO_3H_2 & & CH_2O{\cdot}PO_3H_2 \\ | & & | \\ C{=}O & \rightleftharpoons & CHOH \\ | & & | \\ CH_2OH & & CHO \\ \text{Dihydroxyacetone phosphate} & & \text{3-glyceraldehyde phosphate} \end{array}$$

One molecule of dihydroxyacetone phosphate and one molecule of 3-glyceraldehyde phosphate, in the presence of cozymase or coenzyme I, are converted into one molecule of α-glycerophosphate and one molecule of 3-phosphoglyceric acid.

$$\begin{array}{ccccccc} CH_2O{\cdot}PO_3H_2 & & CH_2O{\cdot}PO_3H_2 & & CH_2O{\cdot}PO_3H_2 & & CH_2O{\cdot}PO_3H_2 \\ | & & | & & | & & | \\ C{=}O & + & CHOH & + H_2O + \xrightarrow[\text{(see page 281)}]{\text{cozymase}} & CHOH & + & CHOH \\ | & & | & & | & & | \\ CH_2OH & & CHO & & CH_2OH & & COOH \\ \text{Dihydroxyacetone phosphate} & & \text{3-glyceraldehyde phosphate} & & \alpha\text{-glycerophosphate} & & \text{3-phosphoglyceric acid} \end{array}$$

In the next step the α-glycerophosphate is hydrolyzed to glycerol and phosphoric acid, and the 3-phosphoglyceric acid is converted first to 2-phosphoglyceric acid and finally to phosphopyruvic acid. The reactions in the latter case are reversible.

$$\begin{array}{ccccc} CH_2O{\cdot}PO_3H_2 & & CH_2OH & & \\ | & & | & & \\ CHOH & + H_2O \rightarrow & CHOH & + & H_3PO_4 \\ | & & | & & \\ CH_2OH & & CH_2OH & & \\ \alpha\text{-glycerophosphate} & & \text{Glycerol} & & \text{Phosphoric acid} \end{array}$$

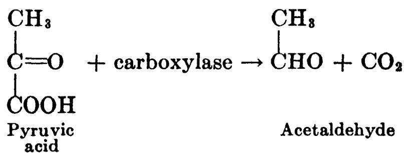
$$\begin{array}{cccccc} CH_2O{\cdot}PO_3H_2 & & CH_2OH & & CH_2 & \\ | & & | & & \| & \\ CHOH & \rightleftharpoons & CHO{\cdot}PO_3H_2 & \rightleftharpoons & CO{\cdot}PO_3H_2 & + H_2O \\ | & & | & & | & \\ COOH & & COOH & & COOH & \\ \text{3-phosphoglyceric acid} & & \text{2-phosphoglyceric acid} & & \text{Phosphopyruvic acid} & \end{array}$$

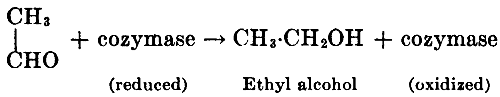
The phosphopyruvic acid is now dephosphorylated by adenosine monophosphate (adenylic acid) to give pyruvic acid and adenosine triphosphate.

$$\begin{array}{ccccc} CH_2 & & & CH_3 & \\ \| & & & | & \\ 2CO{\cdot}PO_3H_2 & + \text{adenylic acid} & + 2H_2O \rightarrow & 2C{=}O & + \text{adenosine triphosphate} \\ | & & & | & \\ COOH & & & COOH & \\ \text{Phosphopyruvic acid} & & & \text{Pyruvic acid} & \end{array}$$

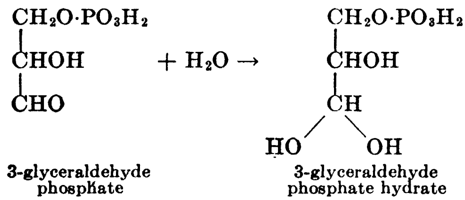
The adenosine triphosphate transfers phosphate to more molecules of hexose to give first hexosemonophosphate, then hexosediphosphate and adenylic acid, and the above series of reactions is repeated.

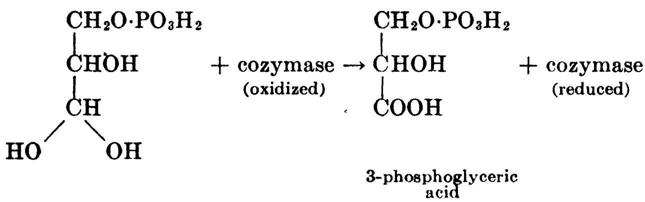
In the final steps the pyruvic acid is decarboxylated to acetaldehyde and carbon dioxide by the enzyme carboxylase. Then the reduced form of cozymase or coenzyme I transfers hydrogen to the acetaldehyde to give ethyl alcohol, and the cozymase becomes oxidized.



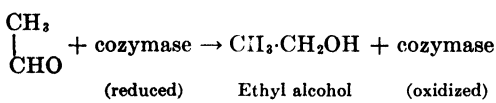


The oxidized cozymase now acts on the 3-glyceraldehyde phosphate, oxidizing it to 3-phosphoglyceric acid, and the cozymase becomes reduced again. The function of the coenzyme is that of a hydrogen acceptor and hydrogen donator to produce reactions of oxidation and reduction.





The reduced cozymase is now capable of acting on more acetaldehyde and reducing it to ethyl alcohol.



The 3-phosphoglyceric acid is then carried through the same series of reactions already outlined to give more acetaldehyde which reacts with more 3-glyceraldehyde phosphate to give 3-phosphoglyceric acid and ethyl alcohol. This cycle continues until fermentation is complete.

The types of reactions involved in the above scheme include (1) the hosphorylation-dephosphorylation reactions in which adenylic acid first ccepts phosphate to become adenosine triphosphate and then transfers hosphate to the hexose; (2) the reversible reactions that include the eaction between hexosediphosphate and 2 molecules of triosephosphate, he reaction between dihydroxyacetone phosphate and 3-glyceraldehyde hosphate, and the reactions between 3-phosphoglyceric acid, 2-phospholyceric acid, and phosphopyruvic acid; (3) the oxidation-reduction eactions in which dihydroxyacetone phosphate and 3-glyceraldehyde hosphate are converted to α-glycerophosphate and 3-phosphoglyceric cid, the 3-glyceraldehyde is oxidized to 3-phosphoglyceric acid accomanied by a reduction of the acetaldehyde to alcohol; and (4) the decaroxylation reaction in which pyruvic acid is decarboxylated to acetaldeyde and carbon dioxide.

In addition to alcohol and carbon dioxide small amounts of *d*-amyl lcohol, isoamyl alcohol, succinic acid, glycerol, and other compounds are lso produced in alcoholic fermentation.

***d*-Amyl and Isoamyl Alcohols.**—A mixture of *d*-amyl and isoamyl lcohols together with traces of other higher alcohols and compound thers obtained from fermented liquors is sometimes referred to as fusel il. Ehrlich showed that *d*-amyl alcohol and isoamyl alcohol are derived rom the amino acids isoleucine and leucine, respectively. These acids nay originate from the constituents of the medium or from the protein f dead and autolyzed yeast cells. The alcohols are produced by a eaminization and a decarboxylation of the amino acids.

$$\underset{\text{Isoleucine}}{\begin{array}{l} CH_3 \diagdown \\ \quad CH\cdot CHNH_2\cdot COOH \\ C_2H_5 \diagup \end{array}} + H_2O \rightarrow \underset{d\text{-amyl alcohol}}{\begin{array}{l} CH_3 \diagdown \\ \quad CH\cdot CH_2OH \\ C_2H_5 \diagup \end{array}} + CO_2 + NH_3$$

$$\underset{\text{Leucine}}{\begin{array}{l} CH_3 \diagdown \\ \quad CH\cdot CH_2\cdot CHNH_2\cdot COOH \\ CH_3 \diagup \end{array}} + H_2O \rightarrow \underset{\text{Isoamyl alcohol}}{\begin{array}{l} CH_3 \diagdown \\ \quad CH\cdot CH_2\cdot CH_2OH \\ CH_3 \diagup \end{array}} + CO_2 + NH_3$$

The amounts of the two alcohols produced depend upon the concenration of the specific amino acids present in the medium; upon the species f yeast employed; and upon the nutritional requirements of the yeast ells. Since the organisms derive their nitrogen by the deaminization of mino acids, the presence of a more easily available source of nitrogen will revent or delay the hydrolysis of amino acids for this purpose. The eaction occurs only in the presence of a fermentable carbohydrate.

Small amounts of alcohols are produced from other amino acids, such as tyrosol from tyrosine and tryptophol from tryptophane.

Succinic Acid.—Succinic acid is produced from glutamic acid during alcoholic fermentation. Ehrlich found that, of all the amino acids added to a fermentation medium, glutamic acid was the only one that gave an increase in the concentration of succinic acid. The most probable course of the decomposition of glutamic acid to succinic acid is the following:

Oxidative deaminization:

$$\underset{\text{Glutamic acid}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CHNH_2{\cdot}COOH} + \tfrac{1}{2}O_2 \rightarrow \underset{\alpha\text{-ketoglutaric acid}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH} + NH_3$$

Decarboxylation:

$$\underset{\alpha\text{-ketoglutaric acid}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH} \rightarrow \underset{\text{Succinic semialdehyde}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CHO} + CO_2$$

Oxidation:

$$\underset{\text{Succinic semialdehyde}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}CHO} + \tfrac{1}{2}O_2 \rightarrow \underset{\text{Succinic acid}}{COOH{\cdot}CH_2{\cdot}CH_2{\cdot}COOH}$$

Succinic acid is produced either from the glutamic acid of the medium or from the proteins of dead and autolyzed yeast cells. As in the case of fusel oil, the production of succinic acid does not occur in the absence of a fermentable carbohydrate.

Glycerol.—Glycerol is prepared commercially chiefly by the saponification of fats and oils in the manufacture of soaps. It is produced in small amounts during the alcoholic fermentation of carbohydrates by yeasts. The yield rarely amounts to more than about 3.8 per cent of the sugar fermented. Neuberg and Reinfurth (1918) were probably the first to show that the yield of glycerol could be greatly increased by adding an appropriate alkali or sodium sulfite to the fermenting mixture.

As has already been shown, alcohol results from a reduction of the intermediary compound acetaldehyde by means of hydrogen from reduced cozymase. If the hydrogen is prevented from reducing the acetaldehyde, an increased yield of glycerol will result. This is accomplished by adding sodium sulfite to the fermenting mixture. The sulfite reacts with the acetaldehyde to produce an addition product preventing it from accepting hydrogen from the reduced cozymase. A second molecule in the fermenting medium, a triose produced from the hexose sugar, acts as a hydrogen acceptor and becomes reduced to glycerol according to the equation,

$$\underset{\text{Acetaldehyde}}{\begin{array}{l} CH_3 \\ | \\ CHO \end{array}} + Na_2SO_3 + H_2O \rightarrow \underset{\substack{\text{Aldehyde-bisulfite}\\ \text{compound}}}{\begin{array}{l} CH_3 \\ | \\ C{-}H \\ |\;\diagdown \\ |\;\;\; OH \\ SO_3Na \end{array}} + NaOH$$

Sodium carbonate reacts with the acetaldehyde in a similar manner to prevent its reduction to alcohol. The yield of glycerol varies, depending upon the amount of sulfite or carbonate added. In the sulfite process the yield of glycerol may be as high as 37 per cent of the sugar fermented.

For further reading on alcoholic fermentation and its chief by-products consult Anderson (1938), Guillaudeu (1937), Harden (1932), Kluyver (1931), Lohmann and Schuster (1937*a*,*b*), Lutwak-Mann and Mann (1935), Meyerhof (1935, 1938), Meyerhof and Kiessling (1933*a*,*b*, 1935*a*,*b*,*c*), Meyerhof and Lohmann (1934), Meyerhof, Lohmann, and Schuster (1936), Michaelis (1935), Michaelis, Moraques—Gonzales, and Smythe (1937), Michaelis and Smythe (1936), Myrbäck (1933), Parnas, Lutwak-Mann, and Mann (1935), Prescott and Dunn (1940), and Stephenson (1939).

BUTYRIC ACID FERMENTATION

The organism involved in this type of fermentation is the anaerobic, spore-forming rod known as *Clostridium butyricum*. It is the same organism that is concerned in the retting of flax and hemp.

The reaction scheme, according to Kluyver and associates (1931), is as follows:

$$\underset{\text{Hexose}}{C_6H_{12}O_6} \rightarrow 2C_3H_6O_3$$

$$\underset{\substack{\text{Methylglyoxal}\\ \text{hydrate}}}{CH_3{\cdot}CO{\cdot}\overset{H}{\underset{OH}{C}}{-}OH} \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Formic acid}}{HCOOH}$$

$$\underset{\text{Formic acid}}{HCOOH} \rightarrow CO_2 + H_2$$

$$\underset{\text{Acetaldehyde}}{2CH_3{\cdot}CHO} + H_2O \rightleftharpoons \underset{\substack{\text{Acetaldehyde}\\ \text{hydrate}}}{CH_3{\cdot}\overset{H}{\underset{OH}{C}}{-}OH} + \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + H_2$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO}$$

$$\underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO} \rightleftharpoons CH_3{\cdot}CH{=}CH{\cdot}\overset{H}{\underset{OH}{C}}{-}OH$$

$$CH_3{\cdot}CH{=}CH{\cdot}\overset{H}{\underset{OH}{C}}{-}OH \rightarrow \underset{\text{Butyric acid}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH}$$

Kluyver believed that the internal mechanism for the conversion of the sugar to methylglyoxal hydrate corresponded to that of alcoholic fermentation.

The important products of this type of fermentation are butyric acid, acetic acid, carbon dioxide, and hydrogen. The theoretical figures given by the above scheme agree remarkably well with the amounts of the final products found in actual tests.

BUTYL ALCOHOL AND ACETONE FERMENTATION

This type of carbohydrate breakdown is characterized by the production of more end products than was noted in the butyric acid fermentation. The two chief products, aside from carbon dioxide, are butyl alcohol and acetone. In addition, smaller amounts of hydrogen, formic acid, acetic acid, butyric acid, ethyl alcohol, and acetylmethylcarbinol have also been recovered.

The organism commonly employed for this purpose is the anaerobe *Clostridium acetobutylicum*. It is a large, motile, spore-forming, Gram-positive rod. The organism is capable of fermenting a large number of carbohydrates and carbohydrate-like compounds.

According to Kluyver (1931) the reaction scheme is as follows:

$$C_6H_{12}O_6 \rightarrow 2C_3H_6O_3$$

(The internal mechanism for this conversion corresponds to that of alcoholic fermentation.)

$$\begin{array}{l} \qquad\qquad\quad H \\ \qquad\qquad\;\, / \\ CH_3\cdot CO\cdot C{-}OH \rightarrow CH_3\cdot CHO + HCOOH \\ \qquad\qquad\;\, \backslash \\ \qquad\qquad\quad OH \end{array}$$

Methylglyoxal hydrate | Acetaldehyde | Formic acid

Dehydrogenation reactions:

$$HCOOH \rightarrow CO_2 + 2H$$

Formic acid

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\quad H \\ \qquad\qquad\qquad\qquad\qquad\;\, / \\ CH_3\cdot CHO + H_2O \rightleftharpoons CH_3\cdot C{-}OH \\ \qquad\qquad\qquad\qquad\qquad\;\, \backslash \\ \qquad\qquad\qquad\qquad\qquad\quad OH \end{array}$$

Acetaldehyde | Acetaldehyde hydrate

$$\begin{array}{l} \qquad\quad H \\ \qquad\;\, / \\ CH_3\cdot C{-}OH \rightarrow CH_3COOH + 2H \\ \qquad\;\, \backslash \\ \qquad\quad OH \end{array}$$

Acetaldehyde hydrate | Acetic acid

Condensation reactions:

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO}$$

$$\underset{\text{Acetaldol}}{CH_3{\cdot}CHOH{\cdot}CH_2{\cdot}CHO} \rightleftharpoons CH_3{\cdot}CH{=}CH{\cdot}C\begin{array}{l}\diagup H\\ —OH\\ \diagdown OH\end{array}$$

$$CH_3{\cdot}CH{=}CH{\cdot}C\begin{array}{l}\diagup H\\ —OH\\ \diagdown OH\end{array} \rightarrow \underset{\text{Butyric acid}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH}$$

$$\underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} \rightarrow CH_3{\cdot}C(OH)_2{\cdot}CH_2{\cdot}COOH$$

$$CH_3{\cdot}C(OH)_2{\cdot}CH_2{\cdot}COOH \rightleftharpoons \underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH} + H_2O$$

$$\underset{\text{Acetoacetic acid}}{CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH} \rightarrow \underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3} + CO_2$$

Hydrogenation reactions:

$$2H \rightarrow H_2$$

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + 2H \rightarrow \underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH}$$

$$\underset{\text{Butyric acid}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}COOH} + 4H \rightarrow \underset{\text{Butyl alcohol}}{CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2OH} + H_2O$$

$$\underset{\text{Acetone}}{CH_3{\cdot}CO{\cdot}CH_3} + 2H \rightarrow \underset{\text{Isopropyl alcohol}}{CH_3{\cdot}CHOH{\cdot}CH_3}$$

The amounts of the end products recovered from a fermenting mixture agree quite closely with theoretical calculations according to the above scheme.

ACETONE AND ETHYL ALCOHOL FERMENTATION

In this type of fermentation the chief products are acetone, ethyl alcohol, and carbon dioxide. Smaller amounts of acetic acid, lactic acid, formic acid, and hydrogen are also produced.

The organism responsible for the acetone and ethyl alcohol fermentation is *Bacillus acetoethylicus*, a motile, aerobic, spore-bearing, Gram-positive rod. Young cells are said to be Gram-negative. The spores are ovoid, terminal, and cause a bulging of the rod. The organism attacks a large number of carbohydrates and carbohydrate-like substances with the production of acid, gas, and other compounds. *B. acetoethylicus* produces about 2 parts of ethyl alcohol to 1 part of acetone. The optimum growth temperature of the organism is about 42 to 45°C. It is probably identical with *B. macerans*.

According to Speakman (1925) the scheme for the fermentation of compounds to acetone and ethyl alcohol is as follows:

Scheme (Part 1):

$$\underset{\text{Hexose}}{C_6H_{12}O_6} \rightarrow 2\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + 2H_2$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + H_2 \rightarrow \underset{\text{Lactic acid}}{CH_3\cdot CHOH\cdot COOH}$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} \rightarrow \underset{\text{Acetaldehyde}}{CH_3\cdot CHO} + CO_2$$

$$\underset{\text{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3\cdot COOH} + \underset{\text{Formic acid}}{H\cdot COOH}$$

$$\underset{\text{Acetaldehyde}}{CH_3\cdot CHO} + H_2 \rightarrow \underset{\text{Ethyl alcohol}}{CH_3\cdot CH_2OH}$$

Scheme (Part 2):

$$\underset{\text{Acetaldehyde}}{CH_3\cdot CHO} + \underset{\text{Acetaldehyde}}{CH_3\cdot CHO} \rightarrow \underset{\text{Acetaldol}}{CH_3\cdot CHOH\cdot CH_2\cdot CHO}$$

$$\left\{\begin{array}{l} \underset{\text{Acetaldol}}{CH_3\cdot CHOH\cdot CH_2\cdot CHO} \\ \\ \underset{\text{Acetaldehyde}}{CH_3\cdot CHO} \end{array}\right. + H_2O \xrightarrow[\text{reduction}]{\text{oxidation}} \begin{array}{l} \underset{\beta\text{-hydroxybutyric acid}}{CH_3\cdot CHOH\cdot CH_2\cdot COOH} \\ \\ \underset{\text{Ethyl alcohol}}{CH_3\cdot CH_2OH} \end{array}$$

$$\left\{\begin{array}{l} \underset{\beta\text{-hydroxybutyric acid}}{CH_3\cdot CHOH\cdot CH_2\cdot COOH} \\ \\ \underset{\text{Acetaldehyde}}{CH_3\cdot CHO} \end{array}\right. + H_2O \xrightarrow[\text{reduction}]{\text{oxidation}} \begin{array}{l} \underset{\text{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} + H_2O \\ \\ \underset{\text{Ethyl alcohol}}{CH_3\cdot CH_2OH} \end{array}$$

$$\underset{\text{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} \rightarrow \underset{\text{Acetone}}{CH_3\cdot CO\cdot CH_3} + CO_2$$

PROPIONIC ACID FERMENTATION

The fermentation of certain carbon compounds to propionic acid is the result of the action of several species of organisms classified under the genus *Propionibacterium*.

Propionibacterium.—The members of this genus are characterized as nonmotile, nonspore-forming, Gram-positive organisms that grow under anaerobic conditions in neutral media as short, diphtheroid rods, sometimes resembling streptococci and under aerobic conditions as long, irregular, club-shaped, and branched rods. The organisms ferment a large number of carbohydrates and polyalcohols with the formation chiefly of propionic acid, acetic acid, and carbon dioxide. The organisms develop very slowly, visible colonies not being discernible until after 5 to 7 days. The cells grow best in yeast extract media containing simple carbohydrates. The optimum temperature for growth is about 30°C. The genus contains 11 well-recognized species.

All the species have been isolated from dairy products and are responsible for the characteristic appearance and flavor of Gruyère (Swiss) cheese.

The first work of importance on the chemistry of the propionic acid fermentation was reported by Fitz (1878). He suggested that the dissimilation of lactic acid occurred according to the equation,

$$\underset{\text{Lactic acid}}{3CH_3{\cdot}CHOH{\cdot}COOH} \rightarrow \underset{\text{Propionic acid}}{2CH_3{\cdot}CH_2{\cdot}COOH} + \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + CO_2 + H_2O$$

Virtanen (1923, 1925) and van Niel (1928) believed that glucose, after preliminary phosphorylation, was converted into two molecules of the C_3 type, one molecule being oxidized to acetic acid and carbon dioxide while two molecules were reduced to propionic acid. van Niel suggested that pyruvic acid was an intermediary product, which was dissimilated according to the equation,

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2O \rightarrow \underset{\text{Pyruvic acid hydrate}}{CH_3{\cdot}C(OH)_2{\cdot}COOH}$$

$$\underset{\text{Pyruvic acid hydrate}}{CH_3{\cdot}C(OH)_2{\cdot}COOH} \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + CO_2 + 2H$$

A number of intermediary compounds have been isolated or detected in fermentation cultures. Foote, Fred, and Peterson (1930), and Fromageot and Tatum (1933) demonstrated the presence of lactic acid. Virtanen and Karström (1931) detected hexosemonophosphate (Robison ester). Pett and Wynne (1933) isolated the compound methyl glyoxal. Wood and Werkman (1934*a*,*b*) showed the presence of pyruvic acid and propionaldehyde and offered more positive proof on the mechanism of propionic acid formation.

In a series of investigations Wood and Werkman (1936, 1938, 1940*a*,*b*), Wood, Stone, and Werkman (1937), and Phelps, Johnson, and Peterson (1939) showed that propionic acid bacteria utilized carbon dioxide during the fermentation of glycerol. In the absence of carbon dioxide the fermentation could be represented by the equation,

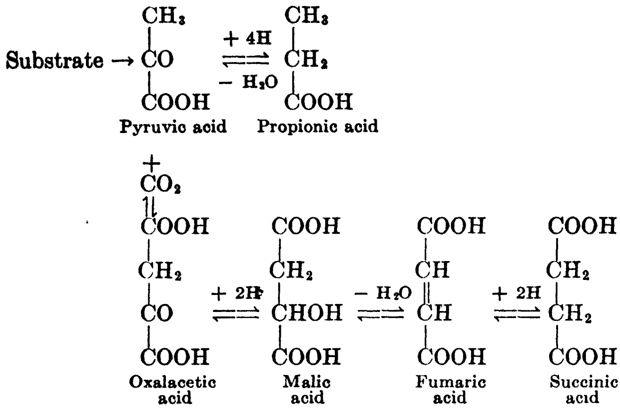
$$\underset{\text{Glycerol}}{CH_2OH{\cdot}CHOH{\cdot}CH_2OH} \rightarrow \underset{\text{Propionic acid}}{CH_3{\cdot}CH_2{\cdot}COOH} + H_2O$$

In the presence of carbon dioxide the formation of propionic acid was accompanied by the appearance of succinic acid. If this were true, the carbon dioxide was converted into succinic acid by combination with a 3-carbon compound.

Ruben, Hassid, and Kamen (1939), Carson and Ruben (1940), Carson, Foster, Ruben, and Kamen (1940), Carson, Foster, Ruben, and Barker (1941), and Wood, Werkman, Hemingway, and Nier (1940) employed radioactive carbon dioxide as an indicator of its utilization by the propionic acid bacteria. On the basis of the information obtained from their tests they concluded that propionic acid and succinic acid contained radioactive carbon only in the carboxyl groups.

Carson, Foster, Ruben, and Barker (1941) suggested the following scheme for the formation of propionic and succinic acids in the presence of C^*O_2:[1]

[1] The symbol C* indicates radioactive carbon.



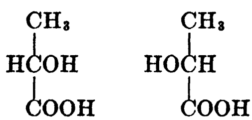
On the basis of their results they came to the following conclusions:

1. Both propionic and succinic acids formed in the presence of C^*O_2 are radioactive.
2. These acids contain the labeled carbon only in the carboxyl (COOH) groups.
3. Pyruvic acid is an intermediate compound in the propionic acid fermentation.
4. A radioactive α-keto acid, besides pyruvic acid, is formed during the fermentation of pyruvic acid in the presence of C^*O_2. This acid contains most, if not all, of the C^* in carboxyl groups.
5. The set of reversible reactions from oxalacetic acid to succinic acid have been found to occur in the propionic acid bacteria.

LACTIC ACID FERMENTATION

Lactic acid was first discovered as one of the products resulting from the souring of milk. It is named after the milk constituent lactose, or milk sugar, which is the precursor of the compound. It is probably the oldest known acid, having been discovered by Scheele in 1780.

The acid produced by bacterial action on carbohydrates is known as fermentation acid. Since lactic acid has an asymmetric carbon atom, it exists in two modifications. These are known as dextrolactic acid and levolactic acid.



Dextrolactic acid Levolactic acid

The organisms that are employed industrially for the production of lactic acid are members of the genus *Lactobacillus*. These organisms differ in the kind of lactic acid produced. Both the dextro and levo forms are always present but one form usually predominates. Some organisms produce more dextro than levo, and others produce more levo than dextro.

If the two acids are produced in equal quantities, the product is inactive by external compensation and is known as a racemic mixture.

Lactobacillus.—The various species consist of large rods, often long and slender and capable of producing lactic acid from carbohydrates and carbohydrate-like compounds. Some species produce gas; others do not. If gas is produced, it consists entirely of carbon dioxide. A number of species are somewhat thermophilic and some are microaerophilic. The genus contains 15 species.

A large number of carbohydrates are employed for the production of lactic acid. Those generally employed are glucose, sucrose, and lactose. Starches of various kinds may be first hydrolyzed to sugars by means of acids or enzymes and then fermented to lactic acid. Molasses and whey are low-priced and excellent sources of carbohydrate for lactic acid production.

The scheme for the fermentation of glucose to lactic acid, according to Nelson and Werkman (1936), is as follows:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Glyceraldehyde}}{CH_2OH{\cdot}CHOH{\cdot}CHO}$$

$$\underset{\text{Glyceraldehyde}}{CH_2OH{\cdot}CHOH{\cdot}CHO} + 2H \rightarrow \underset{\text{Glycerol}}{CH_2OH{\cdot}CHOH{\cdot}CH_2OH}$$

$$\begin{array}{ccc}
\downarrow & & \\
C_3H_6O_3 & \longrightarrow & \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} \\
\downarrow & & \downarrow \\
\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO + CO_2 + 2H} & & \underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH + 2H} \\
\downarrow + 2H & & \downarrow + H_2O \\
\underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH} & & \underset{\text{Acetic acid}}{CH_3{\cdot}COOH + CO_2 + 2H}
\end{array}$$

Acetaldehyde and pyruvic acid are important intermediary compounds. The addition of pyruvic acid to a fermentation medium results in the formation of equimolar quantities of acetic acid, lactic acid, and carbon dioxide.

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + H_2O \rightarrow \underset{\text{Pyruvic acid hydrate}}{CH_3{\cdot}C(OH)_2{\cdot}COOH}$$

$$\underset{\text{Pyruvic acid hydrate}}{CH_3{\cdot}C(OH)_2{\cdot}COOH} \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + CO_2 + 2H$$

$$\underset{\text{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + 2H \rightarrow \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH}$$

ACETIC ACID BACTERIA

Vinegar is a product of the oxidation of alcoholic liquids to acetic acid by certain microorganisms.

Any alcoholic liquid may be employed in the manufacture of vinegar. The fermentation of apple cider to hard cider (alcoholic) by means of

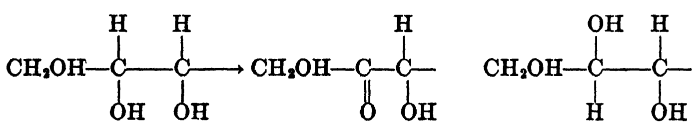
yeasts and then to acetic acid by the specific bacteria yields a product known as cider vinegar. The oxidation of wines yields wine vinegar. The alcoholic fermentation of an infusion of barley malt followed by the acetic fermentation gives a product known as malt vinegar. Sugar vinegar is the result of the alcoholic fermentation of sugar followed by the oxidation of the alcohol to acetic acid. The final product is named after the raw materials used in its manufacture. All vinegars contain about 4 per cent of acetic acid.

When any alcoholic liquid is exposed to the air, a film appears on its surface. At the same time the liquid becomes sour, owing to the oxidation of the alcohol to acetic acid. The film is composed of a viscous gelatinous substance, or zooglea, in which are embedded many bacteria. It is commonly known as mother of vinegar because a small portion of this material is capable of acting as a starter when added to more alcoholic liquid.

The bacteria present in a zoogleal mass are classed under the genus *Acetobacter*. They are the so-called acetic acid bacteria. These organisms are dependent upon a plentiful supply of oxygen for growth and multiplication. That is why their activities are confined to the surface of alcoholic liquids.

Acetobacter.—The cells are rod-shaped, frequently in chains, and motile or nonmotile. Usually they grow on the surface of alcoholic liquids as obligate aerobes, securing growth energy by the oxidation of alcohol to acetic acid. They are also capable of utilizing many other carbonaceous compounds. Elongated, filamentous, club-shaped, swollen, and even branched cells may occur as involution forms. The genus contains 12 species.

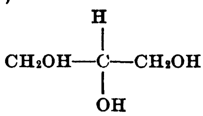
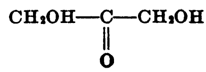
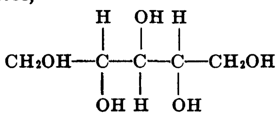
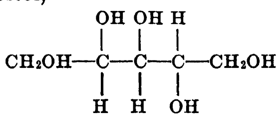
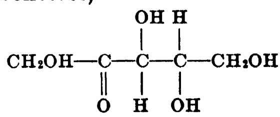
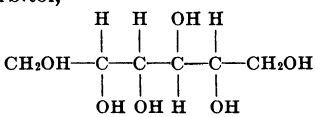
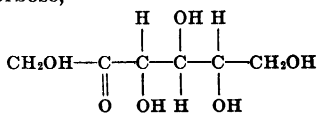
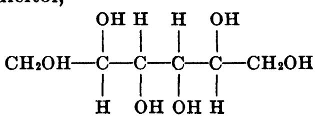
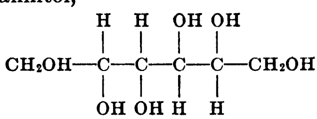
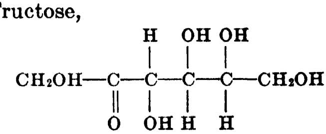
The acetic acid bacteria are capable of oxidizing other primary alcohols and aldehydes to carboxylic acids and secondary alcohols to ketones. Bertrand (1904) found that *Acetobacter xylinum* (sorbose bacillus) was capable of oxidizing a secondary alcohol group of sorbitol to the corresponding ketone sugar sorbose. He noted that the organism oxidized some alcohols but failed to attack others. On careful examination of his results he concluded (1) that only secondary alcohol groups are attacked; (2) that the group oxidized must be on the second carbon atom; and (3) that the (OH) groups on the second and third carbon atoms must be adjacent to each other for oxidation to occur.



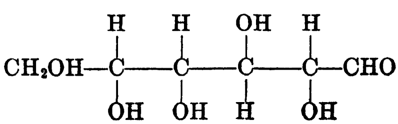
Attacked   Ketone sugar   Not attacked

The effect of the configuration of a number of higher alcohols on fermentability by *A. xylinum* is given in Table 41.

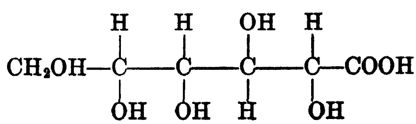
TABLE 41.—EFFECT OF CONFIGURATION ON OXIDATION BY *Acetobacter xylinum*.

Alcohol	Oxidation Product
Ethylene glycol, $CH_2OH{\cdot}CH_2OH$	Not oxidized
Glycerol, 	Dihydroxyacetone, 
Xylitol, 	Not oxidized
Arabitol, 	Araboketose, 
Sorbitol, 	Sorbose, 
Dulcitol, 	Not oxidized
Mannitol, 	Fructose, 

Hermann and Neuschul (1931) noted an exception to the above rule. They found that the aldehyde group of *d*-glucose was oxidized to a carboxyl group to give *d*-gluconic acid, even though the (OH) groups on the second and third carbon atoms are not adjacent to each other.



d-glucose



d-gluconic acid

Aerobic Oxidation of Alcohol.—The aerobic oxidation of alcohol to acetic acid is generally believed to occur according to the following scheme:

1. Oxygen acts as a hydrogen acceptor, converting alcohol to acetaldehyde.

$$\underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH} + O \rightarrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O$$

2. Acetaldehyde becomes hydrated.

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O \rightarrow \underset{\text{Hydrated acetaldehyde}}{CH_3{\cdot}C\begin{array}{l} \diagup H \\ -OH \\ \diagdown OH \end{array}}$$

3. Oxygen accepts two hydrogen atoms from the hydrated acetaldehyde to give acetic acid.

$$\underset{\text{Hydrated acetaldehyde}}{CH_3{\cdot}C\begin{array}{l} \diagup H \\ -OH \\ \diagdown OH \end{array}} + O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + H_2O$$

Anaerobic Oxidation of Alcohol.—In the absence of oxygen 1 molecule of acetaldehyde may act as a hydrogen acceptor for a second hydrated molecule of acetaldehyde. This results in the formation of 1 molecule of acetic acid and 1 of ethyl alcohol from 2 molecules of acetaldehyde, according to the following scheme:

1. Hydration of acetaldehyde.

$$\underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + H_2O \rightarrow \underset{\text{Hydrated acetaldehyde}}{CH_3{\cdot}C\begin{array}{l} \diagup H \\ -OH \\ \diagdown OH \end{array}}$$

2. Cannizzaro reaction.

$$\underset{\text{Hydrated acetaldehyde}}{CH_3{\cdot}C\begin{array}{l} \diagup H \\ -OH \\ \diagdown OH \end{array}} + \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Ethyl alcohol}}{CH_3{\cdot}CH_2OH}$$

Neuberg and Windisch (1925) believed that ethyl alcohol was first oxidized to acetaldehyde, which in turn was dismutated to equimolar

quantities of ethyl alcohol and acetic acid according to the foregoing reactions. Alternate oxidation and dismutation continued until all the ethyl alcohol was converted into acetic acid.

The acetic acid concentration in the final solution is diluted to 4 per cent of acetic acid to give commercial vinegar. In addition to acetic acid, traces of esters also produced in the fermentation are largely responsible for the pleasant odor and flavor of vinegar.

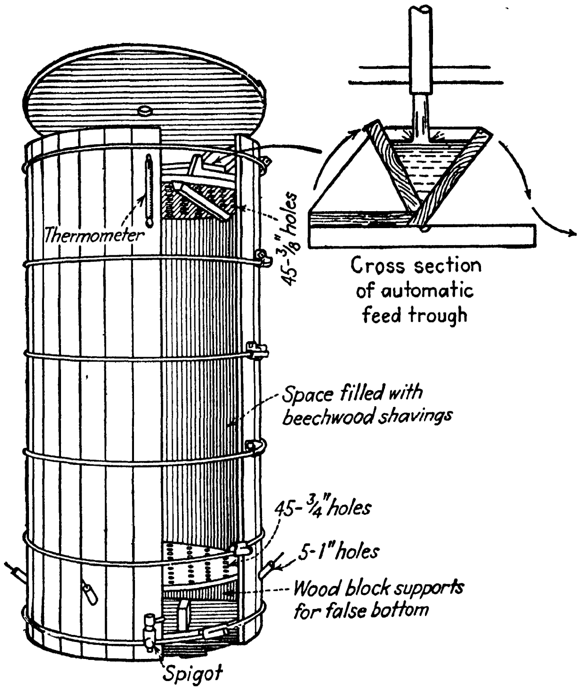


FIG. 134.—Generator used in the quick vinegar process. (*From Prescott and Dunn, Industrial Microbiology.*)

Vinegar may lose its strength on standing. This is due to the oxidation of the acetic acid to carbon dioxide and water by some species of *Acetobacter.*

$$\underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + 2O_2 \rightarrow 2CO_2 + 2H_2O$$

This reaction takes place only in the presence of considerable oxygen. The oxidation may be prevented by storing vinegar in well-filled, tightly stoppered bottles or by the destruction of the organisms by pasteurization.

Methods of Manufacture.—Two general methods are employed in the manufacture of vinegar: (1) the Orleans method and (2) the quick method.

Orleans Method.—This is the oldest commercial method known for the preparation of vinegar. Barrels or vats are perforated near the top to permit free entrance of air and then filled about two-thirds full with a mixture composed of 2 parts of vinegar and 3 parts of wine. The wine may be raw or pasteurized, the latter being preferable since it greatly reduces the percentage of abnormal fermentations. The acetic acid bacteria grow better in a strongly acid medium. For this reason vinegar

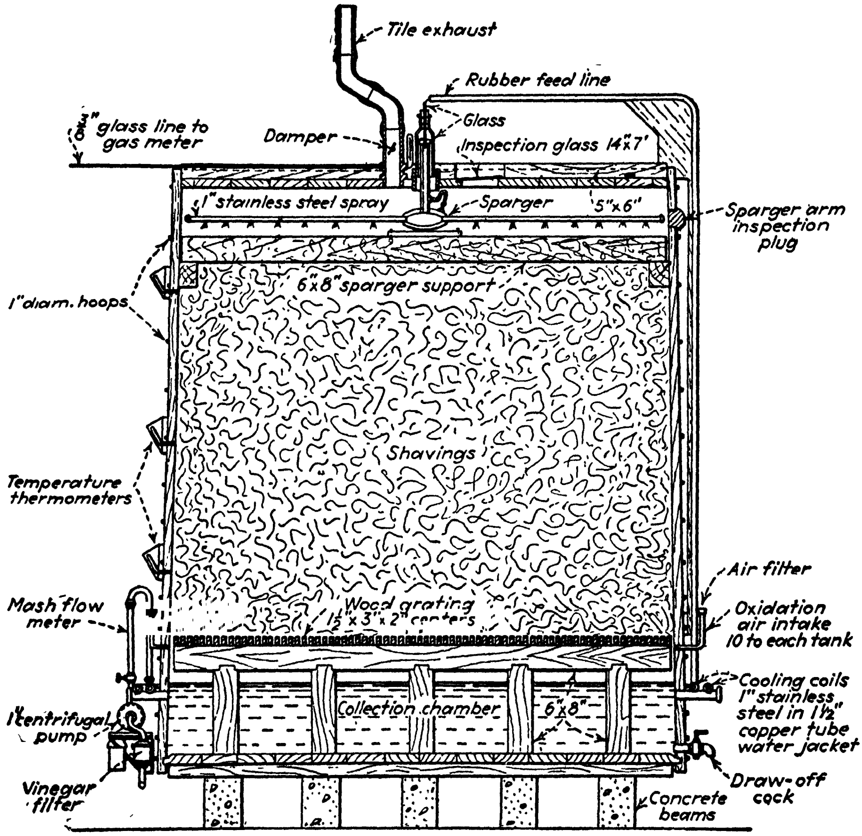


FIG. 135.—Cross section of the Frings generator. (*From Prescott and Dunn, Industrial Microbiology.*)

is added to the wine to speed up the reaction and at the same time to check the growth of undesirable organisms. At definite intervals some of the vinegar is drawn off and fresh wine added. In this manner the process becomes continuous.

Quick Method.—The quick method depends upon the use of large wooden tanks or generators with perforated bottoms through which air enters (Fig. 134). These tanks are filled with beechwood shavings, charcoal, etc., to give greater aeration of the alcoholic liquid by increasing

the surface area. The shavings or charcoal are first soaked with a culture of acetic acid bacteria. Then the alcoholic solution is sprayed at the top of the tank and allowed to trickle over the surface of the shavings. By the time the liquid reaches the bottom of the tank, the alcohol becomes oxidized to acetic acid by the organisms present on the shavings. The temperature is kept at about 35 to 38°C. After the completion of the process the vinegar is drawn off at the bottom of the tank. A generator 10 ft. in diameter and 20 ft. high is capable of producing from 80 to 100 gal. of vinegar per day.

Many modifications of the above generator are employed for the manufacture of vinegar. One of these, known as the Frings generator, possesses many advantages (Fig. 135). It is cheap and simple to operate. It produces vinegars having higher concentrations of acetic acid than those produced by other methods. Since the tank is smaller it utilizes less space. The generator is airtight, thus avoiding loss of vinegar or alcohol by evaporation.

FERMENTATION OF CARBOHYDRATES

Carbohydrates and compounds of a similar nature are added to culture media to serve as readily available sources of energy. Carbohydrates are also incorporated in culture media for another very important purpose. Organisms vary considerably in their ability to ferment various carbohydrates. Some bacteria are able to attack a carbohydrate and produce acid and gas; others are able to produce acid but not gas; still others fail to ferment the compound (Fig. 136). Such information is of considerable value in the identification and classification of organisms.

It is not clearly understood why an organism ferments one aldose sugar and not another having the same empirical formula. The sugars differ only in the arrangement of H atoms and OH groups around carbon atoms. There is no method for determining beforehand whether or not a particular organism is capable of fermenting a given carbohydrate. This can be determined only by making the test. As Kendall, Bly, and Haner (1923) stated, " . . . the carbohydrate, to be utilizable, must possess a stereoconfiguration which is compatible with a corresponding asymmetry of the protoplasm of the microbe." Emil Fischer in his studies on the chemistry of the carbohydrates expressed the above relationship as that of a key fitting its particular lock.

Sternfeld and Saunders (1937) concluded from their studies that any change from the structure of the aldose sugars resulted in a decreased frequency of fermentation of the derivatives. Robbins and Lewis (1940) in their studies on the fermentation reactions of a large number of organisms came to the same general conclusions. They found that *d*-gluconic, *d*-mannonic, and *d*-galactonic acids, as well as *d*-sorbitol, *d*-mannitol, and

dulcitol, were fermented by fewer organisms than the corresponding aldoses. Similarly, the dicarboxylic mucic and *d*-saccharic acids were attacked less frequently than the corresponding monocarboxylic *d*-galactonic and *d*-gluconic acids. Georgi and Ettinger (1941) obtained similar results in their studies on several species of *Rhizobium.* In general, mono- and disaccharides were more frequently attacked than tri- and polysaccharides and the sugar acids.

Organisms of the colon group and related species ferment glucose with the production of such compounds as acetic, formic, succinic, and lactic acids, ethyl alcohol, acetylmethylcarbinol, 2:3-butylene glycol, carbon

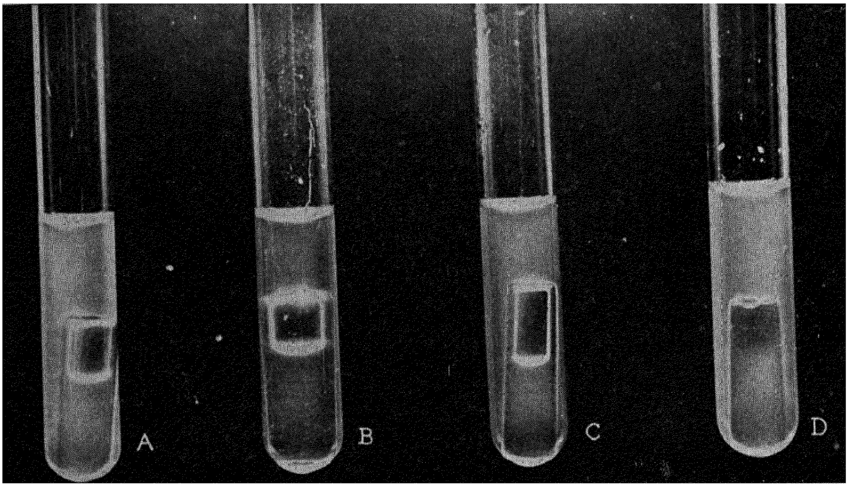


FIG. 136.—Fermentation of carbohydrates. *A*, glucose; *B*, lactose; *C*, sucrose, all inoculated with *Escherichia communior.* Acid and gas produced. *D*, sucrose inoculated with *E. coli.* No acid or gas.

dioxide, and hydrogen. These organisms produce probably the most common types of bacterial fermentations.

The scheme for the fermentation of glucose by *Escherichia coli,* as reported by Kluyver (1931), is given in Fig. 137.

The compounds account for about 99 per cent of the carbon of the fermented glucose. A similar analysis on the same medium fermented by *Aerobacter aerogenes* does not account for such a high percentage of the carbon of the glucose. It has been shown that the discrepancy is due to the production by *Aerobacter aerogenes,* but not by *E. coli,* of the compound 2:3-butylene glycol. The scheme for the fermentation is given in Fig. 138.

It may be concluded that the breakdown of glucose by members of the *Escherichia* and *Aerobacter* groups involves three types of fermentations: (1) the typical fermentation of the nonphosphorylated glucose

to succinic acid, (2) a true alcoholic fermentation of the phosphorylated glucose, and (3) the formation of acetaldehyde and formic acid, with the decomposition of the latter to carbon dioxide and hydrogen. One of

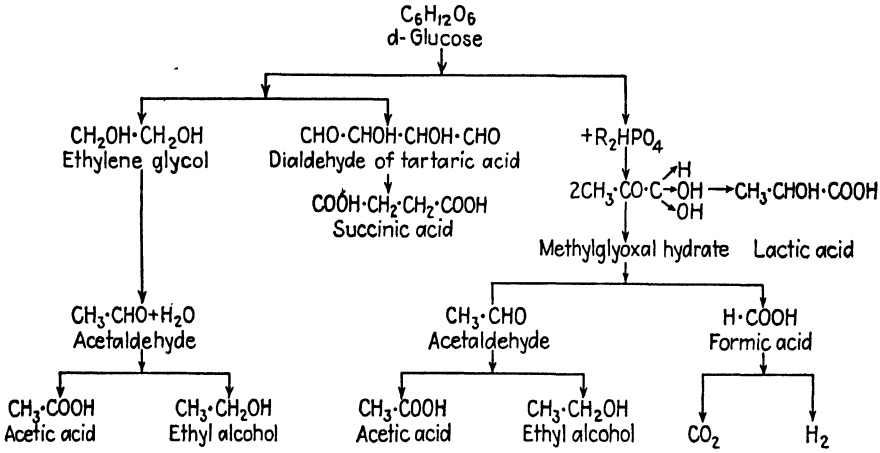


FIG. 137.—Scheme for the fermentation of glucose by *Escherichia coli.*

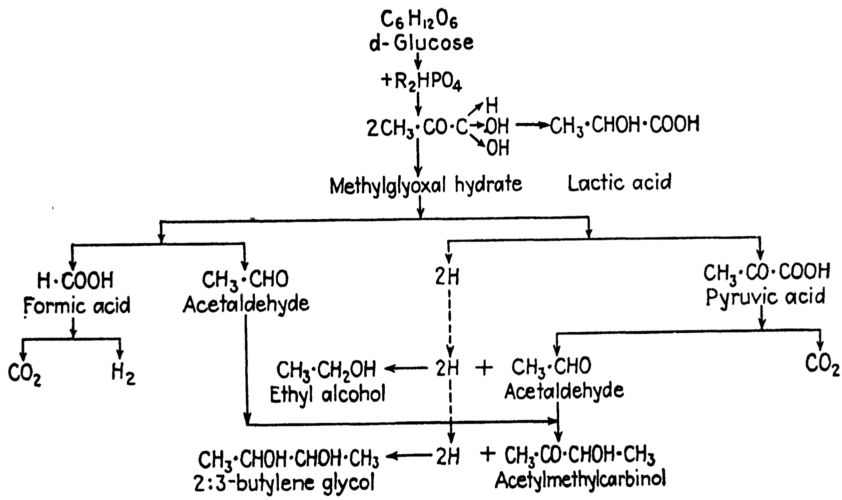


FIG. 138.—Scheme for the fermentation of glucose by *Aerobacter aerogenes.*

these three types of fermentations predominates, depending upon the species of organism involved.

SOME ROUTINE FERMENTATION REACTIONS EMPLOYED FOR THE IDENTIFICATION OF BACTERIA

The ability of an organism to ferment a particular carbohydrate is determined by incorporating an appropriate indicator in a liquid or a

solid medium. Gas production is detected by placing an inverted vial in a carbohydrate broth medium to trap the gas as it is evolved.

Litmus Carbohydrate Media.—Litmus is a weakly staining dye and is employed only as an indicator. Since it is not a delicate detector of changes in acidity or alkalinity, it has been largely replaced by the more sensitive and brilliant sulfonephthalein indicators.

Litmus possesses an important advantage over the newer indicators in that it is sensitive to decolorization by some organisms. It functions

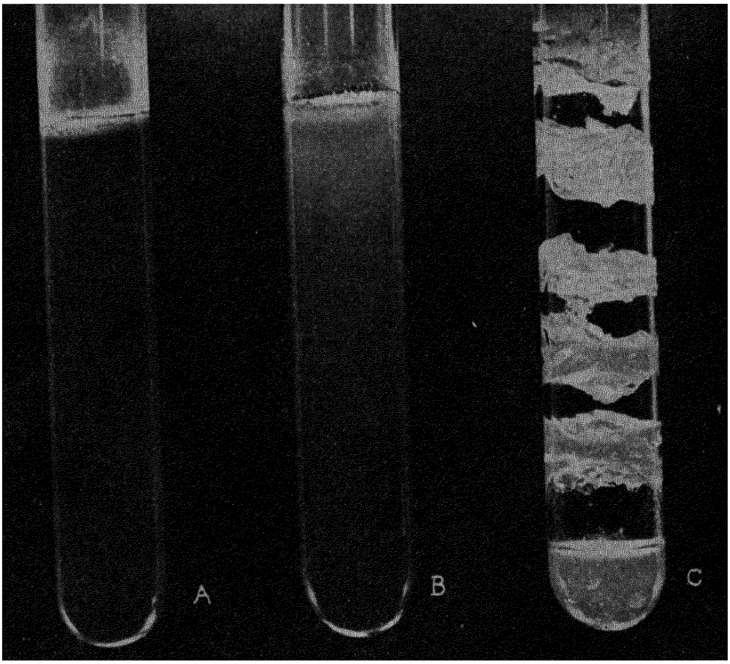


FIG. 139.—Lactose litmus agar. *A, Bacillus subtilis; B, Staphylococcus aureus; C, Escherichia coli. Bacillus subtilis* and *Staphylococcus aureus* produce neither acid nor gas. *Escherichia coli* produces acid and gas with the result that the agar is separated into rings and the litmus is decolorized.

as an oxidation-reduction system. The dye is colored when oxidized, and colorless when reduced. The decolorization of the indicator is first noted in the deeper layers of culture media where the dissolved oxygen is soon exhausted. The indicator present in the surface layer of culture media seldom, if ever, becomes completely decolorized because the organisms find it more convenient to utilize the oxygen of the atmosphere.

The fermentation of a tube of deep carbohydrate agar results in a splitting of the agar by the escaping gas. In some cases the agar splits in the form of disks with a layer of gas separating each disk of agar (Fig. 139). Under some conditions the gas pressure may be sufficient to

force some of the agar disks, together with the cotton stopper, out of the tube.

Bromocresol Purple Carbohydrate Media.—The fermentative ability of an organism can be determined very easily by streaking a loopful of the culture over the surface of nutrient agar containing carbohydrate and indicator. Fermentation of the carbohydrate results in an increase in the hydrogen-ion concentration of the agar. The visible result is a change in the color of the indicator.

If only a few well-isolated colonies appear on the plate, acid production remains confined to areas immediately surrounding the colonies. This results in a color change in the vicinity of each colony without affecting the agar free from colonies. The contrast in the colors in the acid and alkaline or neutral regions of the agar becomes very striking.

In the presence of a mixed culture composed of two organisms, only one of which is capable of fermenting the carbohydrate, isolations of two different colonial forms may be successfully realized. This is possible only if the culture is diluted highly before being streaked and, also, if the two organisms are present in approximately equal numbers.

Bromocresol purple is frequently used as the indicator in carbohydrate media. This indicator is especially valuable for bacteriological work because of its brilliant colors at different hydrogen-ion concentrations and its resistance to decolorization by bacterial action. The sensitive range of the indicator is from pH5.2 to 6.8. It is yellow at pH5.2 and purple at pH6.8. The pK value, *i.e.*, the point at which the dye is 50 per cent dissociated, is pH6.3. Its pH range makes it suitable for the detection of bacterial fermentations in solid carbohydrate media.

Reversal of Reaction.—It sometimes happens that the acid reaction in carbohydrate media turns alkaline with respect to the indicator employed, if the plates or tubes are stored for several days. This may be explained on the basis of (1) a lack of sufficient carbohydrate and (2) the oxidation of the organic acids to carbonates.

If a medium contains an insufficient amount of carbohydrate, an organism will not be able to produce its limiting hydrogen-ion concentration. Under these conditions the organism will continue to multiply by utilizing the nitrogenous constituents for both structure and energy. This results in the production of alkaline products with the consequent reversal in the reaction of the medium.

Reversal in the reaction of a medium sometimes occurs even in the presence of an amount of carbohydrate sufficient for an organism to produce its limiting hydrogen-ion concentration. It was shown by Ayers and Rupp (1918) that under these conditions simultaneous acid and alkaline reactions occur. The carbohydrates are first fermented to organic acids, which are in turn oxidized to carbonates. The alkaline

carbonates are responsible for the reversal in the reaction of the medium. The alkaline changes do not occur after all of the carbohydrate has been converted to acid but run simultaneously with acid production.

ENSILAGE FERMENTATION

The preparation of ensilage affords a rather simple method for the preservation of foods for stock animals. Any farm crop is satisfactory for ensilage production provided it contains sufficient sugar to furnish the required amount of acid for its preservation.

The plants are cut into small pieces and packed into silos. Sufficient moisture must be present in the cut pieces, otherwise water is added. Since the surface of the plants contains the required kinds of organisms, it is not necessary to inoculate the material. Counts have shown that there are normally present from 10,000 to 400,000,000 bacteria per gram of plant tissue.

Because of the presence of carbohydrates in the plant sap, the changes that occur are fermentative rather than putrefactive. Within a few hours the aerobic organisms utilize the free oxygen present in the silo. The changes that follow are anaerobic rather than aerobic in character. After a period of about 48 to 72 hr. from 70 to 80 per cent of the gas present in the silo is carbon dioxide. Considerable heat is generated during the fermentation process. When fermentation is active, each gram of the silage may contain as many as 2,000,000,000 organisms. Each cubic centimeter of the silage juice may show a count as high as 1,000,000,000 bacteria. Fermentation is complete after a period of about 1 month. The organisms responsible for the fermentation are certain streptococci and several species of the genus *Lactobacillus*. The organisms produce chiefly lactic acid, acetic acid, butyric acid, propionic acid, and small amounts of alcohol. The quantity and proportion of each varies, depending upon the kinds of plants used.

TOBACCO FERMENTATION

Tobacco is cured in order to improve its aroma and texture. During this treatment about 28 per cent of the nicotine is lost, accompanied by an increase in citric acid.

The leaves are stacked in piles and allowed to ferment. During this stage heat is generated, the temperature going as high as 60°C. Oxygen is consumed and carbon dioxide, ammonia, and other volatile substances are released. The flavors of tobaccos are improved by moistening the leaves with sugars, sirups, malt extract, honey, etc., which are responsible for the development of aromatic esters and other compounds. After the completion of the fermentation the leaves are dried and then used in the manufacture of various kinds of tobaccos. The course of the fermenta-

tion appears to be greatly affected by variations in the composition of the tobacco leaf. Failure of certain crops to undergo a satisfactory fermentation has been responsible for great losses in the industry.

The nature of the chemical changes that occur during fermentation are not clearly understood. As stated by Reid, McKinstry, and Haley (1938*a*),

> Three theories have been advanced to account for the chemical changes occurring during the fermentation process by workers who have studied the problem. The first theory advanced maintained that the reactions were purely of an oxidative character and were not catalyzed by enzymes. That the fermentation of cigar leaf may be explained in this manner is no longer the belief of those familiar with the process, but the literature of recent years on the subject of the fermentation of other types of tobacco implies acceptance of this theory. The second theory advanced ascribed the chemical reactions as due to the activities of microorganisms. Although 50 years have passed since the inception of this theory, the support accorded it in recent years has been vague and somewhat contradictory. The third theory attempts to account for the chemical reactions during fermentation on the basis of catalysis by plant enzymes, and this appears to be accepted by the majority of those engaged in the processing of cigar-leaf tobacco at the present time. Its supporters have vigorously attacked the bacterial theory and left it but few adherents.

Reid, McKinstry, and Haley (1938*b*) made a study of the bacterial flora of tobacco and reported that the predominant forms upon cured leaf were bacteria of the *Bacillus megatherium* group and molds of the genera *Penicillium* and *Aspergillus*. A satisfactory fermentation was found to be associated with a rapid increase in numbers of organisms of the *Micrococcus candicans* type and of the *B. subtilis-mesentericus-vulgatus* group. The predominant types upon cured leaf played little if any part in a satisfactory fermentation. Viable fungi disappeared during the early stages of the fermentation and bacteria of the *B. megatherium* group failed to show any significant increase in number during the process. Some cellulose-decomposing species of the genus *Clostridium* were occasionally encountered on cured tobacco. Since these organisms are anaerobic in character, they multiply when the oxygen concentration is sufficiently reduced. The cellulose-digesting species are capable of multiplying at 55°C. and are usually responsible for the rotting that sometimes occurs during the fermentation process.

For further reading on bacterial fermentations consult Anderson (1938), Arzberger, Peterson, and Fred (1920), Bernhauer (1938, 1939), Brown, Wood, and Werkman (1938), Burton (1937), Butlin (1936), Cruess (1938), Davis (1939), Hann, Tilden, and Hudson (1938), Hansen (1935), Kendall (1923), Kendall and Yoshida (1923), Kluyver (1935),

May and Herrick (1930), McDaniel, Woolley, and Peterson (1939), Nelson and Werkman (1935, 1936), Pederson (1938), Pett and Wynne (1933), Pan, Peterson, and Johnson (1940), Prescott and Dunn (1940), Prescott and Proctor (1937), Reid (1940), Rogers (1936), Sjolander, Langlykke, and Peterson (1938), Smith and Claborn (1939), Snell and Mitchell (1941), Speakman (1923), Rogers and Whittier (1940), Stephenson (1939), Tatum, Peterson, and Fred (1935), Underkofler, Christensen, and Fulmer (1937), Underkofler and Hunter (1938).

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CHAPTER XVI

DIFFERENTIATION AND CLASSIFICATION OF BACTERIA

GENERAL CONSIDERATIONS

The First International Microbiological Congress, which met in Paris in 1930, voted to follow the rules of nomenclature adopted by international congresses of botany and zoology insofar as it was possible to do so. This recommendation was approved by the plenary sessions of the Microbiological Congress and of the Botanical Congress. The congress voted also to establish an International Committee on Bacteriological Nomenclature, with permanent secretaries in London, England, and Geneva, N. Y.

From 1930 to the present time, the committee has been actively engaged in producing a more stable nomenclature and workable classification of bacteria. Some of its recommendations have been accepted by the Second International Congress of Microbiology, which met in London in 1936. These recommendations have been incorporated in the descriptive portion of the 1939 edition of "Bergey's Manual of Determinative Bacteriology."

The most important sections of the International Rules of Botanical Nomenclature, of interest to the bacteriologist, are grouped as follows:

1. General considerations and guiding principles.
2. Categories of taxonomic groups, and the terms denoting them.
3. Names of taxonomic groups.

Under these three sections may be grouped such considerations as (1) fixity of names and avoidance of unscientific terms, (2) general principles of nomenclature, and (3) general principles of taxonomy.

Fixity of Names.—In order that reference may be made to an organism it must be given a name. Every organism has at least one name to distinguish it from other kinds of bacteria.

Two kinds of names are given to the different kinds of organisms. Each organism possesses a scientific name, which is more or less international in meaning. In addition an organism may possess one or more common names, which are of local interest. The common names are responsible for considerable confusion in bacteriology. For this reason all organisms should be referred to by their scientific names, which are supposed to be the same in any language.

General Principles of Nomenclature.—The method followed in naming organisms was first introduced by the Swedish botanist Karl von Linné. The method is known as the binomial system of nomenclature.

Each kind of organism or species has two names. The first word is the name of the genus and the second that of the species. The name of the genus is usually taken from the Latin, or rarely, the Greek, language. It is a noun and is always capitalized. It may be masculine, or feminine, or neuter. Examples of the three genders are: Bacillus (masculine); Sarcina (feminine); and Bacterium (neuter).

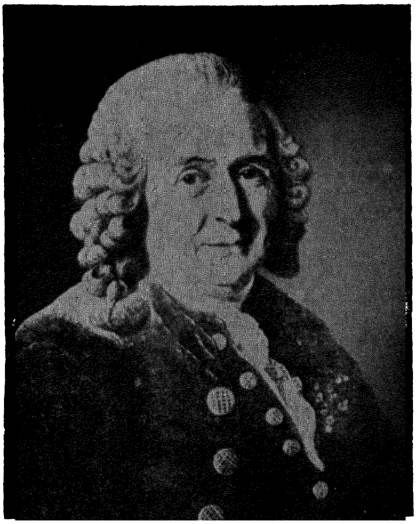

Fig. 140.—Karl von Linné.

The second or species name is generally an adjective and is not capitalized. It may be capitalized if it is derived from a proper noun but it is not necessary. For the sake of consistency it is better to capitalize only the name of the genus.

Sometimes a species is subdivided into varieties. These are also given Latin designations. For example, *Mycobacterium tuberculosis* has been subdivided into the two varieties, *M. tuberculosis* var. *hominis* (human tuberculosis) and *M. tuberculosis* var. *bovis* (tuberculosis of cattle).

General Principles of Taxonomy.—The term taxonomy may be defined as the classification of plants and animals according to their natural relationships. It is compounded from the two Greek words τάξις, an arrangement, order, and νομος, a law.

A satisfactory development of taxonomy is dependent upon a sound nomenclature. Regardless of whether bacteriologists will ever be able to agree on the exact classification to be employed, they should agree on some of the fundamental characteristics necessary for the development of a satisfactory bacteriological classification.

Each kind of plant or animal is referred to as a species (plural, species). The term is defined in various ways but in bacteriology it is usually stated to be the lowest member of a classificatory system. A bacterial species is a plant that occupies a place in a classification between the genus and the variety. Since the differences between varieties are often very difficult to recognize, it is the species that to the untrained observer usually seem to represent the simplest distinct assemblages or kinds in the plant or animal kingdoms.

The first described specimen of a species is spoken of as the type of the species. It is used as the type species for all other species regarded as sufficiently like the type to be placed together in the same group or genus.

A group of closely related species is spoken of as a genus (plural, genera). It ranks between the family and the species. Closely related genera, then, are grouped into families. Finally, closely related families are grouped into orders.

Differentiating Bacteria.—Hundreds of species of bacteria have been recognized and studied. They represent probably only a small percentage of the total number of species in existence. Since bacteria are so small and undifferentiated, it is an exceedingly difficult matter to identify and classify them.

During the early years of bacteriology organisms were classified entirely on the basis of morphology. Only a few species were recognized and their classification was comparatively a simple matter. The morphological characters employed include size and shape of an organism; arrangement of the cells; presence or absence of well-defined capsules; presence or absence of spores; size and shape of the spore and position in the cell; presence, number, and arrangement of flagella; irregular forms; presence or absence of characteristic metachromatic granules; acid fastness; Gram reaction and other differential staining procedures; cultural and colonial characteristics; etc. As more and more species were recognized, the problems of classification became increasingly more difficult. At the present time it would be a hopeless task to attempt to identify and classify bacteria entirely on morphology because there are so few characteristics on which to base a classification.

Higher plants are differentiated almost entirely on the basis of morphology. One tree may be easily distinguished from another by differences in such characteristics as the size, shape, and color of the tree; size, shape, and color of the leaves and seeds; etc. In the case of bacteria it is quite evident that the problems of classification are more difficult owing to the fact that such minute organisms are comparatively simple spheres, rods, and spirals.

It soon became apparent that a classification based entirely on morphology alone was inadequate and that more characteristics were necessary. Physiological reactions were, therefore, introduced into the newer classifications. They include such reactions as temperature relations; chromogenesis or pigment pro[illegible] of a change in the reaction of the environment on growth; production of [illegible]ion of hydrogen sulfide; relation to oxygen; reduction of nitrate to nitrite and even to ammonia, and finally to free nitrogen; fermentation of carbohydrates; etc. At the present time physiological reactions are prob

ably more important than morphological differences in the classification of bacteria.

Sometimes it is necessary to resort to animal inoculation and serological reactions to separate similarly appearing and reacting organisms. Serological methods will be discussed in Chap. XXIV, Infection and Immunity.

Bacteria are placed in the class Schizomycetes (fission fungi). This class is composed of seven orders. The common or true bacteria fall

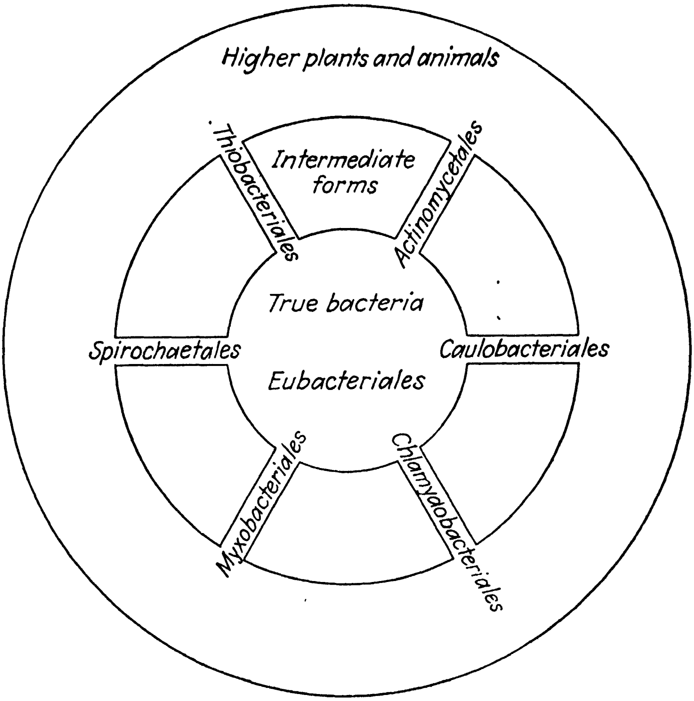

Fig. 141.—The seven orders of bacteria in the class Schizomycetes.

into the order Eubacteriales. Organisms placed into the other six orders have characteristics intermediate between the true bacteria and higher plants and animals. This is shown in Fig. 141.

The abridged classification[1] that follows is based on the work of the International Committee on Bacteriological Nomenclature.

Class: *Schizomycetes*.—This class is characterized as typically unicellular plants. The cells are usually small and relatively primitive in organization. The cells are spherical, cylindrical, spiral, or filamentous. In the latter case there may be false or more frequently true branching. The cells are often united into groups,

[1] Reprinted by permission from "Bergey's Manual of Determinative Bacteriology," by Bergey, Breed, Murray, and Hitchens, published by The Williams & Wilkins Company, Baltimore, 1939.

families, or filaments; occasionally in the latter showing some differentiation among the cells, simulating the organization seen in certain of the blue-green, filamentous algae. Multiplication typically by cell fission. Endospores are formed by some species of the Eubacteriales; conidia by some of the filamentous forms. Chlorophyll is not produced by any of the bacteria (bacterio-chlorophyll is found in a few species). Many forms produce pigments of other types. The cells may be motile by means of flagella; some of the forms intergrading with the protozoa are flexuous. A few filamentous forms show oscillatory movement similar to that of certain blue-green algae (as Oscillatoria).

Order I. *Eubacteriales.* These are simple and undifferentiated forms, without true branching. They occur as spheres, short or long straight rods, or as curved rods. The cells may be motile or nonmotile. Endospore formation occurs in some species. Some species form pigment, others store reserve materials as volutin, glycogen, or fat. Sulfur and iron are not stored as visible particles.

Order II. *Actinomycetales.* The cells are usually elongated, frequently filamentous and with a tendency toward the development of branches, in some genera giving rise to the formation of a definite branched mycelium. Cells frequently show swellings, clubbed or irregular shapes. No pseudoplasmodium observed. No deposits of free sulfur, iron, or bacteriopurpurin. Endospores are not produced but conidia are developed in some genera. Cells are usually Gram-positive and nonmotile. Some species are parasitic in animals or plants. As a general rule the cells are strongly aerobic and oxidative. Complex proteins frequently required for nutrition. Growth on culture media often slow. Some genera show mold-like colonies. The organisms are not water forms.

Order III. *Chlamydobacteriales.* The organisms are filamentous, alga-like, typically water forms, and ensheathed. They may be unbranched, show true branching, or false branching. It is possible that the true branching species may eventually prove to be aquatic species of Actinomycetales. False branching arises from lateral displacement of the cells of the filament within the sheath, which gives rise to a new filament, so that the sheath is branched while the filaments are separate. The sheath may be composed entirely of iron hydroxide, or of an organic matrix impregnated with iron or may be entirely organic. Conidia and motile "swarmers" may be developed, but never endospores. Sulfur granules or bacteriopurpurin are absent. Mature cells or filaments are not protozoa-like.

Order IV. *Caulobacteriales.* The bacteria are nonfilamentous, growing characteristically upon stalks. The cells are asymmetrical in that gum, ferric hydroxide, or other material is secreted from one side or one end of the cell to form the stalk. The cells multiply typically by transverse fission. In one family multiplication takes place by longitudinal fission and by budding. In some species the stalks may be very short or absent, the cells being connected directly to the substrate or to each other by holdfasts. Cells occur singly or in pairs, never in chains or filaments; not ensheathed. The cells are typically aquatic in habitat; some may be parasitic in animals.

Order V. *Thiobacteriales.* The cells may be spherical, short or long straight rods, short spirals, or filamentous. They contain either granules of free sulfur, or bacteriopurpurin, or both, and usually grow best in the presence of hydrogen sulfide. The cells are plant-like, not protozoan-like, and do not produce a pseudoplasmodium or a highly developed resting stage. Spores are rarely or never produced. The cells do not have a sheath.

Order VI. *Myxobacteriales.* The cells are mold-like. They develop as a colony (pseudoplasmodium or swarm) consisting of slender, relatively flexible, elongate

rods. The cells move together as an advancing mass by the excretion of a slime. Flagella have not been observed. The fruiting bodies may consist of numerous spores that develop by a shortening of the rods, or of cysts in the interior of which lie more or less shortened rods. Various types of fruiting bodies are observed, sometimes sessile, frequently stalked, usually colored yellow or red.

The organisms are usually cultivated on dung media. Most species are found on dung or isolated from the soil. One species is aquatic, parasitic on Cladophora.

Order VII. *Spirochaetales.* The organisms are protozoan-like in certain characters. The cells are usually slender, flexuous spirals. Multiplication takes place by transverse division. There is no conclusive evidence of longitudinal division. Motility is often characteristic but without polarity. Endospores are absent.

An abridged classification of the organisms grouped under the seven orders into families and genera is as follows:

Order I. *Eubacteriales.* Characteristics given on page 362.

Family I. *Nitrobacteriaceae.* Organisms are usually rod-shaped, sometimes spherical. Some species have a single polar flagellum; others are nonmotile. Sometimes branched involution forms are produced. The organisms are autotrophic. They depend upon hydrogen, methane, carbon monoxide, ammonia, nitrite, sulfur, or thiosulfates. The cells are nonparasitic and usually occur in water or soil.

Genus I. *Nitrobacter.* The cells are rod-shaped, nonmotile, do not grow readily on organic media, and oxidize nitrites to nitrates.

Genus II. *Nitrosomonas.* The cells are rod-shaped. Some species are motile, possessing polar flagella, while others are nonmotile. They secure growth energy by oxidizing ammonia to nitrites. Growth in media containing organic matter usually scanty or absent.

Genus III. *Nitrosococcus.* These are large, spherical organisms, showing no growth on ordinary media. They oxidize ammonia to nitrites in the soil and in appropriate culture media.

Genus IV. *Hydrogenomonas.* Organisms are short rods capable of growing in the absence of organic matter. They secure energy by the oxidation of hydrogen to water.

Genus V. *Methanomonas.* Cells are monotrichous, short rods capable of growing in the absence of organic matter. They secure energy by the oxidation of methane to carbon dioxide and water.

Genus VI. *Carboxydomonas.* Cells are rod-shaped and autotrophic. They secure energy by the oxidation of carbon monoxide to carbon dioxide.

Genus VII. *Thiobacillus.* These are small, rod-shaped organisms, which derive their energy from the oxidation of sulfides, thiosulfates, or elementary sulfur, forming sulfur, persulfates, and sulfates under acid and alkaline conditions. They derive their carbon from carbon dioxide or from carbonates and bicarbonates in solution. Some species are obligate; others are facultative autotrophic. One species is anaerobic.

Family II. *Rhizobiaceae.* The cells are rod-shaped, motile, or nonmotile. The motile species have a single polar or lateral flagellum, or 2-4 peritrichous flagella. They show a tendency to be Gram-negative. The organisms utilize glucose and sometimes other sugars without producing organic acids in appreciable quantities.

Genus I. *Rhizobium.* The members of this genus are aerobes capable of producing nodules on the roots of leguminous plants. The symbiosis results in

the fixation of atmospheric nitrogen available to the plant. The rods are Gram-negative, motile when young, and commonly changing to bacteroids upon (*a*) artificial culture media containing alkaloids, glucosides, or in which acidity is increased, or (*b*) during symbiosis within the nodule. Organisms are heterotrophic and grow best at 25°C. Nitrates slowly reduced to nitrites; nitrites not utilized.

Genus II. *Chromobacterium.* The cells are aerobic and produce a violet, chromoparous pigment soluble in alcohol but not in chloroform.

Genus III. *Alcaligenes.* The rods are peritrichous to monotrichous or nonmotile and occur generally in the intestinal canal, in decaying materials, dairy products, and soil. Acetylmethylcarbinol is not formed. Organic acids not produced from carbohydrates.

Family III. *Pseudomonadaceae.* The rods are elongate and vary from straight to more or less spirally curved. Cell division is transverse, never longitudinal. Cells are nonflexuous and do not produce spores. The rods are sometimes nonmotile but usually motile by means of polar flagella. They are Gram-negative. The organisms occur in water and soil. Some species are parasitic in plants and animals.

Genus I. *Vibrio.* The rods are short, bent, rigid, single, or united into spirals. They are motile by means of one (rarely two or three) polar flagellum. Cells are facultative anaerobic, usually Gram-negative, and nonspore forming. Many species liquefy gelatin and are active ammonifiers. They occur in water. A few species are parasitic.

Genus II. *Cellvibrio.* The cells are slender, slightly curved, with rounded ends, and show deeply staining granules, which appear to be concerned in reproduction. They are motile with one polar flagellum. The organisms oxidize cellulose to oxycellulose. Growth on ordinary culture media is weak.

Genus III. *Cellfalcicula.* The cells are short rods or spindle-shaped with pointed ends. Metachromatic granules are present. Old cultures show coccoid forms. Cells are motile by means of one polar flagellum. They oxidize cellulose to oxycellulose. Growth on ordinary culture media is weak.

Genus IV. *Spirillum.* The rods are rigid and of varying thickness, length, and pitch of spiral, forming either long screws or portions of a turn. They are usually motile by means of a tuft of polar flagella. The flagella may appear at one or both poles and the number varies greatly and is difficult to determine. They occur in water and putrid infusions.

Genus V. *Pseudomonas.* The organisms produce a water-soluble pigment which diffuses through the medium as a green, blue, or yellowish-green pigment. Some species are motile; others are nonmotile. Gram-negative. They are principally water and soil bacteria.

Genus VI. *Phytomonas.* The rods may be yellow or white and most species are motile. Motility is due to either monotrichous or lophotrichous flagella. Many species form a green fluorescent water-soluble pigment. Most species are Gram-negative. The organisms produce a necrosis in plants.

Genus VII. *Protaminobacter.* These are motile and nonmotile Gram-negative rods. They attack one or more of the lower alkylamines, and grow moderately or poorly on ordinary peptone agar. These organisms are especially endowed to attack substances containing the group $HN\langle{}^{C}_{C}$.

Genus VIII. *Mycoplana.* The rods are motile and show branching. They are capable of using aromatic compounds, such as phenol, as a source of energy. The organisms occur in the soil. Gram-negative.

Family IV. *Acetobacteriaceae.* The cells are rod-shaped, but frequently with elongated, branched, or swollen forms. They are capable of oxidizing alcohol to acetic acid.

Genus I. *Acetobacter.* The cells are rod-shaped, frequently in chains, motile by means of polar flagella or nonmotile. They usually grow on the surface of alcoholic solutions as obligate aerobes and secure their energy by the oxidation of alcohol to acetic acid. They are also capable of utilizing many other carbonaceous compounds, such as sugar and acetic acid. The cells may be elongated, filamentous, club-shaped, swollen, and even branched.

Family V. *Azotobacteriaceae.* These are large rods or oval cells which utilize free nitrogen.

Genus I. *Azotobacter.* The cells are large rods or even cocci, sometimes almost yeast-like in appearance. They are dependent for energy primarily upon the oxidation of carbohydrates. They may be motile or nonmotile. Motility is due to one or a tuft of polar flagella. They are obligate aerobes, usually growing as a film on the surface of the culture medium. The organisms are capable of fixing atmospheric nitrogen when grown in solutions containing carbohydrates and deficient in combined nitrogen. The cells grow best in media poor in nitrogen.

Family VI. *Micrococcaceae.* The cells are spherical in their free condition and somewhat elliptical during division. Division occurs in two or three planes. If the cells remain in contact after division, they are flattened in the plane of division, and occur singly, in pairs, tetrads, packets, or irregular masses. Motility is rare. Endospores are probably absent. They produce an abundant surface growth on ordinary media. Their metabolism is complex, usually involving the utilization of amino acids and carbohydrates. Many species form a lemon-yellow, orange, or red pigment. They are aerobes, facultative anaerobes, and anaerobes. The species are generally Gram-positive.

Genus I. *Micrococcus.* The cells occur in plates or irregular masses but never in long chains or packets. They are generally Gram-positive. Growth on agar is usually abundant. Some species do not form a pigment; others produce yellow or, less commonly, orange or red pigment. Glucose broth becomes slightly acid, lactose broth generally neutral. Gelatin is frequently liquefied but not rapidly. They are facultative parasites or saprophytes.

Genus II. *Staphylococcus.* The cells occur singly, in pairs, and in irregular groups, rarely in packets. They are usually Gram-positive. Growth is fair to good on the surface of artificial media. As a rule carbohydrates are fermented with the formation of acid. Gelatin is commonly liquefied. Nitrates may or may not be reduced to nitrites. Some produce hemolysis on blood agar. A white or orange or, less commonly, a lemon-yellow pigment is formed. The organisms are usually parasitic.

Genus III. *Gaffkya.* The organisms occur in the animal body and in special media as tetrads; in ordinary culture media they occur in pairs and irregular masses. They are aerobic to anaerobic and Gram-positive. The cells are parasitic.

Genus IV. *Sarcina.* The organisms are saprophytes and facultative parasites. Division occurs, under favorable conditions, in three planes, producing regular packets. They are usually Gram-positive. Growth on agar is abundant,

usually with the formation of yellow or orange pigment. Glucose broth is slightly acid; lactose broth is generally neutral. Gelatin is frequently liquefied. Nitrates may or may not be reduced to nitrites.

Family VII. *Neisseriaceae.* These are strict parasites, some species failing to grow or growing poorly on ordinary culture media. They are aerobes, facultative anaerobes, and anaerobes. The organisms grow best at 37°C.; some species show no growth at 20°C. Growth is fairly abundant in serum media. The cells occur in pairs and in masses. They stain Gram-negative.

Genus I. *Neisseria.* The organisms are paired Gram-negative cocci with adjacent sides flattened. They are anaerobes, facultative anaerobes, and aerobes. The organisms are found on mucous membranes or invade the blood stream and localize in the tissues, joints, or meninges of mammals. They show limited biochemical activity. Few carbohydrates are utilized. Indole is not produced. Nitrates are not reduced to nitrites. Catalase is produced abundantly.

Genus II. *Veillonella.* The cells are very small cocci, occurring in masses, rarely in pairs or short chains. They are nonmotile and Gram-negative. The cells are undifferentiated and united by an interstitial substance of an ectoplasmic nature. The organisms are generally plasmolyzed in hypertonic 5 per cent salt solution in a few hours. They are anaerobic.

Family VIII. *Parvobacteriaceae.* The cells are small, motile, or nonmotile rods, which grow well on media containing body fluids. They are Gram-negative. The organisms usually do not liquefy gelatin. They are not active in the fermentation of carbohydrates. They are usually parasitic on warm-blooded animals, infection in some cases taking place by penetration of organisms through the mucous membranes or skin.

Genus I. *Pasteurella.* The rods are small Gram-negative, ovoid to elongated, and show bipolar staining by special methods. They are aerobic and facultative and require low oxidation-reduction potential on primary isolation. Fermentative powers are slight. Lactose is not fermented. Gelatin is not liquefied. Milk is not coagulated. They are parasitic on man, animals, and birds.

Genus II. *Malleomyces.* The rods are short, with rounded ends, sometimes forming threads and showing a tendency toward branching. They may be motile or nonmotile and Gram-negative. They show a tendency to bipolar staining. Milk is slowly coagulated. Gelatin may be liquefied. The cells grow well on blood serum and other body fluid media. The organisms are specialized for a parasitic life.

Genus III. *Brucella.* These are minute rods with many coccoid cells. They are motile or nonmotile, and Gram-negative. Gelatin is not liquefied. Acid or gas not produced from carbohydrates. They are parasitic, invading animal tissue, producing infection of the genital tract, the mammary gland, or the lymphatic tissues, the respiratory and intestinal tracts. The cells are pathogenic for various species of domestic animals and man.

Genus IV. *Hemophilus.* The cells are minute, rod-shaped, sometimes thread-forming and pleomorphic. They are nonmotile. The organisms are strict parasites growing best (or only) in the presence of hemoglobin and in general requiring blood serum, ascitic fluid, or certain growth accessory substances. They stain Gram-negative.

Genus V. *Noguchia.* The rods are small, slender, and Gram-negative. They are present in the conjunctiva of man and animals affected by a follicular type

of disease. The growth is mucoid. The organisms are motile, flagellated, and encapsulated. They are aerobic and facultative anaerobic. The optimum temperature for growth is 28 to 30°C.

Genus VI. *Dialister*. Cells are minute, rod-shaped, occur singly, in pairs, and short chains. They are nonmotile and strictly parasitic. Growth occurs only under anaerobic conditions in media containing fresh, sterile tissue or ascitic fluid.

Family IX. *Lactobacteriaceae*. The cells are rods and cocci, occurring singly, in pairs, and in chains. Carbohydrates are readily fermented with the production of lactic acid and some volatile acid. Some types also produce carbon dioxide and ethyl alcohol from glucose and mannitol from levulose. They are rarely motile. Gelatin is rarely liquefied. Nitrates are not utilized. Pigment, if produced, is yellow, orange-red, or rusty brown. The cells are aerobic to anaerobic. They do not grow well on the surface of agar media. Cells are Gram-positive.

Genus I. *Diplococcus*. The organisms are parasitic, growing poorly or not at all on artificial media. The cells are usually in pairs, somewhat elongated, encapsulated, and sometimes in chains. They are Gram-positive. Fermentative powers are high, most strains forming acid from glucose, lactose, sucrose, and inulin. Aerobic species are bile-soluble.

Genus II. *Streptococcus*. The cells are spherical or ovoid, rarely elongated into rods, and occur in short or long chains, or in pairs. They are never arranged in packets and do not form zoogloeal masses. Cells stain Gram-positive. Capsules are not marked as a rule but are well developed at times. Growth tends to be slight on artificial media and some species are aided by the addition of native proteins. Isolated colonies are small and translucent. They may be effuse, convex, or mucoid. Cultures are found that produce a rusty red growth in deep agar stabs. Certain strains form a yellow or orange pigment in starch broth. Action on blood is variable but characteristic changes are produced by some species. Little surface growth is produced in stab cultures. Various carbohydrates are fermented with dextrorotary lactic acid as the dominant product. Carbon dioxide, volatile acids, and other volatile compounds are produced in small quantities, if at all, from carbohydrate fermentation. Nitrates are not reduced to nitrites and inulin is rarely attacked. Most species are aerobic and facultative anaerobic. Many species are normally parasitic and some are highly pathogenic. None are soluble in bile. The strictly anaerobic streptococci, some of which produce gas and foul odors, are not yet completely defined and they may merit being separated in a new genus.

Genus III. *Leuconostoc*. The cells normally occur as spheres. They are nonmotile and Gram-positive. Under certain conditions, such as in acid fruit and vegetables, the cells may lengthen and become pointed or even elongated into a rod.

They grow on ordinary media, but growth is enhanced by the addition of yeast cells, extract of yeast, or other vegetable tissues. Generally produce a limited amount of acid. They rarely curdle milk. Mannitol is produced from levulose. By-products of the fermentation of glucose include carbon dioxide, lactic acid, acetic acid, and ethyl alcohol. Approximately one-fourth of the dextrose fermented is converted to carbon dioxide.

Levolactic acid is always produced and sometimes dextrolactic acid. Certain types grow with a characteristic slime formation in sucrose media. Ordinarily the amount of soluble nitrogen in the medium is not increased even after a long period of incubation.

Genus IV. *Lactobacillus*. The cells are rods, often long and slender. They always produce lactic acid from carbohydrates. When gas is formed, it is carbon dioxide without hydrogen. A number of species are somewhat thermophilic. As a rule they are microaerophilic.

Genus V. *Propionibacterium*. The cells are nonmotile, nonspore-forming, and Gram-positive. They grow under anaerobic conditions in neutral media as short diphtheroid rods, sometimes resembling streptococci. Under aerobic conditions they grow as long, irregular, club-shaped, and branched cells. Metachromatic granules demonstrable with Albert's stain. Lactic acid, carbohydrates and polyalcohols, fermented with the formation of propionic and acetic acids and carbon dioxide. As a rule they are strongly catalase-positive, sometimes weakly so. The cells show a strong tendency towards anaerobiosis. Development is very slow, macroscopically visible colonies generally not discernible in less than 5 to 7 days. Nutritional requirements complex. Optimum temperature is 30°C.

Family X. *Enterobacteriaceae*. The organisms are Gram-negative rods and widely distributed in nature. Many of them are parasitic in animals; some produce diseases in plants. They grow well on artificial media. All species attack carbohydrates, forming acid or acid and gas. All produce nitrites from nitrates. When motile the flagella are peritrichous.

Genus I. *Escherichia*. Cells are nonspore-forming, Gram-negative short rods, ferment glucose and lactose with the formation of acid and gas, and grow aerobically. They occur commonly in the intestinal tract of animals. The species are widely distributed in nature. They are methyl red positive and Voges-Proskauer negative. Carbon dioxide and hydrogen are produced in approximately equal volumes from glucose.

Genus II. *Aerobacter*. The rods are nonspore-forming, Gram-negative, grow aerobically, and ferment glucose and lactose with the production of acid and gas. They are widely distributed in nature. The methyl-red test is negative and the Voges-Proskauer test is positive. Two or more times as much carbon dioxide as hydrogen is formed from glucose. Trimethyleneglycol is not produced from glycerol by anaerobic fermentation. Citric acid is utilized as the only source of carbon.

Genus III. *Klebsiella*. The members are short rods, somewhat plump with rounded ends, and occur mostly singly. They are nonmotile, Gram-negative, and encapsulated. The cells ferment a number of carbohydrates with the formation of acid and gas. Nitrates are reduced to nitrites. They are encountered principally in the respiratory tract of man. The organisms are aerobic and grow well on ordinary culture media.

Genus IV. *Erwinia*. The members are plant pathogens. They invade the plants and produce local lesions. They are usually motile with peritrichous flagella. Most species are Gram-negative.

Genus V. *Serratia*. The organisms are small, aerobic, Gram-negative, peritrichous rods, which produce a characteristic red pigment. White to rose-red strains that lack brilliant colors are common. Nitrates are reduced to nitrites, gelatin is rapidly liquefied, milk is coagulated and digested, and blood serum is liquefied. Typical species produce carbon dioxide and frequently hydrogen from glucose, and other sugars; also acetic, formic, succinic, and lactic acids, acetylmethylcarbinol, and 2:3 butylene glycol.

Genus VI. *Proteus*. The organisms are highly pleomorphic rods. Filamentous, curved rods, and involution forms are common. They are Gram-negative

and generally actively motile by means of peritrichous flagella. Produce amoeboid colonies, etc., on moist media and decompose proteins. Glucose and generally sucrose are fermented but not lactose. Voges-Proskauer reaction usually negative. Urea is decomposed.

Genus VII. *Salmonella.* Organisms are usually motile but nonmotile forms occur. Numerous carbohydrates are attacked with the formation of acid, and usually gas. Lactose, sucrose, and salicin are not attacked. Indole is not formed. Gelatin is not liquefied.

Genus VIII. *Eberthella.* The organisms are Gram-negative, motile rods. A number of carbohydrates are attacked with the formation of acid but no gas. Acetylmethylcarbinol is not formed.

Genus IX. *Shigella.* The cells are rods, nonmotile, and Gram-negative. A number of carbohydrates are attacked with the formation of acid but no gas. Acetylmethylcarbinol is not produced.

Family XI. *Bacteriaceae.* The cells are rod-shaped and do not produce spores. Some are motile and some nonmotile. Their metabolism is complex, amino acids are utilized, and generally carbohydrates. This is a heterogeneous collection of genera whose relationships to each other and to other groups are not clear.

Genus I. *Listerella.* The rods are small, without spores, Gram-negative, and motile by means of one long terminal flagellum. They are aerobic to microaerophilic. The organisms grow freely on ordinary media. Certain carbohydrates are attacked. They are pathogenic parasites, the infection being characterized by a monocytosis.

Genus II. *Microbacterium.* The rods are small, nonmotile, nonspore-forming, and Gram-positive. Lactic acid is produced from carbohydrates. May be a transition group to types that liquefy gelatin and do not form acid from carbohydrates. Nitrates are reduced to nitrites. Surface growth on media is good. Catalase is produced.

Genus III. *Kurthia.* The rods are long and occur in evenly curved chains. They are Gram-positive and motile with peritrichous flagella. A proteus-like growth is produced on media. Carbohydrates and gelatin are not attacked. Hydrogen sulfide is not formed.

Genus IV. *Cellulomonas.* The cells are small rods with round ends, nonspore-forming, motile with peritrichous flagella, or nonmotile, occur in the soil and have the property of digesting cellulose. Growth on ordinary culture media is often not vigorous. They are Gram-negative.

Genus V. *Achromobacter.* The rods do not produce pigment and occur in water and soil. They are motile by means of peritrichous flagella or nonmotile. The Gram stain is negative.

Genus VI. *Flavobacterium.* The cells are rods of medium size, occur in water and soil, and form a yellow to orange pigment on culture media. Attack on carbohydrates is feeble and occasionally form acid from hexoses but no gas. The organisms are generally Gram-negative, nonmotile, or motile by means of peritrichous flagella.

Genus VII. *Actinobacillus.* The rods are medium-sized, aerobic, Gram-negative, and frequently show much pleomorphism. Coccus-like forms are frequent. Acid without gas is usually produced from carbohydrates. They grow best, especially when freshly isolated, under increased carbon dioxide tension. The cells are pathogenic for animals, and some species occasionally attack man. The outstanding characteristic of the group is the tendency to

form aggregates in tissues or culture, which resemble the so-called sulfur granules of actinomycosis.

Genus VIII. *Bacteroides.* The organisms are motile or nonmotile rods, do not produce spores, are obligate anaerobes and Gram-negative. They may or may not require enriched culture media.

Genus IX. *Fusobacterium.* The rods usually show tapering ends, are Gram-negative, anaerobic, and usually nonmotile. They stain with more or less distinct granules.

Genus X. *Bacterium.* This genus includes those species which are nonspore-forming and rod-shaped and whose position in the system of classification is not definitely established.

Family XII. *Bacillaceae.* The organisms are rods, produce spores, are Gram-positive, and flagella, when present, are generally peritrichous. Protein media often decomposed actively.

Genus I. *Bacillus.* Bacteria are rod-shaped, sometimes in chains, aerobic, and nonmotile or motile by means of peritrichous flagella. Endospores are formed. The cells are generally Gram-positive. The genus is subdivided into 10 groups.

Genus II. *Clostridium.* Rods are anaerobic or microaerophilic and often parasitic. The cells are frequently enlarged at sporulation, producing clostridium or plectridium forms.

Order II. *Actinomycetales.* Characteristics given on page 362.

Family I. *Mycobacteriaceae.* The cells grow as straight or slightly curved rods, or slender filaments, and are frequently irregular in form with only slight and occasional branching. The organisms often stain unevenly (showing variations in staining reactions within the cell). The organisms are Gram-positive, nonmotile, aerobic, and do not form conidia.

Genus I. *Corynebacterium.* The rods are slender, often slightly curved, with a tendency to club and pointed forms, and with branching forms in old cultures. The organisms are Gram-positive, nonmotile, usually aerobic and do not produce endospores. They show barred and uneven staining. The cells are not acid-fast. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when the cells divide.

Genus II. *Mycobacterium.* The rods are slender and stain with difficulty, but when once stained are acid-fast. Cells sometimes show swollen, clavate, or cuneate forms, and occasionally even branched forms. Growth on media is slow for most species. The cells are aerobic. Several species are pathogenic for animals.

Family II. *Actinomycetaceae.* The rods are branched and filamentous, sometimes forming a mycelium. Sometimes conidia are present. Some species are parasitic and some are soil forms.

Genus I. *Leptotrichia.* Organisms grow as thick, long, straight or curved filaments, unbranched, frequently clubbed at one end and tapering to the other. The cells are Gram-positive when young. Filaments fragment into short, thick rods. They are anaerobic or facultative. Aerial hyphae or conidia are not formed. They are parasites or facultative parasites.

Genus II. *Erysipelothrix.* Organisms are rod-shaped with a tendency to the formation of long filaments, which may show branching. The filaments may also thicken and show characteristic granules. The cells are nonmotile, Gram-positive, microaerophilic, and do not produce spores. They are usually parasitic.

Genus III. *Proactinomyces.* The organisms grow as slender filaments or rods, frequently swollen, and occasionally branched. Generally form, in the first

stage of growth, very small mycelia which early assume the appearance of bacterium-like growths. Shorter rods and coccoid forms are found in older cultures. Conidia are not formed. They stain readily, occasionally showing a slight degree of acid-fastness. They are nonmotile, nonspore-forming, aerobic, and Gram-positive. The colonies are similar in gross appearance to those of the genus *Mycobacterium*. Paraffin, phenol, and *m*-cresol are frequently utilized as a source of energy.

Genus IV. *Actinomyces*. The organisms grow in the form of a much-branched mycelium, which may break up into segments and function as conidia. They are sometimes parasitic with clubbed ends of radiating threads conspicuous in lesions in the animal body. Some species are microaerophilic or anaerobic. The organisms are nonmotile.

Order III. *Chlamydobacteriales*. Characteristics given on page 362.

Family I. *Chlamydobacteriaceae*. Characters for the family are the same as for the order.

Genus I. *Sphaerotilus*. The organisms are attached, colorless threads, showing false branching, though this may be rare in some species. Filaments consist of rod-shaped or oval cells, surrounded by a firm sheath. Multiplication occurs both by nonmotile conidia and by motile "swarmers," the latter with lophotrichous flagella.

Genus II. *Clonothrix*. Filaments are attached and show false branching as in *Sphaerotilus*. Sheaths are organic, encrusted with iron or manganese, broader at the base, and tapering toward the tip. The cells are colorless and cylindrical. Reproduction takes place by spherical conidia formed in chains by transverse fission of cells. Conidia formation is acropetal and limited to short branches of the younger portion of the filaments.

Genus III. *Leptothrix*. The filaments are cylindrical, colorless cells, with a sheath at first thin and colorless, later thicker, yellow or brown, and encrusted with iron oxide. The iron may be dissolved by dilute acid, whereupon the inner cells show up well. Multiplication takes place by division and abstraction of cells and by motile cylindrical swarmers. True branching may occur.

Genus IV. *Crenothrix*. The filaments are not branched, and attached to a firm substrate, showing differentiation of base and tip. Sheaths are plainly visible, thin and colorless at the tips, thick and encrusted with iron at the base. Cells are cylindrical to spherical, dividing in three planes to produce spherical, nonmotile conidia.

Order IV. *Caulobacteriales*. Characteristics given on page 362.

Family I. *Nevskiaceae*. The bacteria are stalked, the long axis of the rod-shaped cells being set at right angles to the axis of the stalk. Stalks are lobose, dichotomously branched, and composed of gum. Multiplication of cells takes place by transverse binary fission. They grow in zoogloea-like masses in water or in sugar vats.

Genus I. *Nevskia*. Description of the genus is the same as for the family.

Family II. *Gallionellaceae*. The bacteria are stalked, the long axis of the rod-shaped cells being set at right angles to the axis of the stalks. Stalks are slender, twisted bands, dichotomously branched, composed of ferric hydroxide, which completely dissolves in dilute hydrochloric acid. Multiplication takes place by transverse binary fission. They grow in iron-bearing waters.

Genus I. *Gallionella*. Description of the genus is the same as for the family.

Family III. *Caulobacteriaceae*. The bacteria are stalked, the long axis of the elongated cells coinciding with the long axis of the stalks. Stalks are slender, flagellum-like, often attached to the substrate by a button-like holdfast, and

unbranched. Multiplication is by transverse binary fission. The outermost cell of a pair may form a stalk before cell division is complete. They are periphytic, growing upon submerged surfaces.

Genus I. *Caulobacter.* Description of the genus is the same as for the family.

Family IV. *Pasteuriaceae.* The bacteria are stalked with spherical or pear-shaped cells. If the cells are elongated, the long axis of the cell coincides with the axis of the stalk. Stalks may be very short or absent but, when present, are usually very fine and at times arranged in whorls attached to a common holdfast. Cells multiply by longitudinal fission or by budding or by both. They are mostly periphytic, one species is parasitic.

Genus I. *Pasteuria.* The cells are pear-shaped and attached to each other or to a firm substrate by holdfasts secreted at the narrow end. They multiply by longitudinal fission and by budding of spherical or ovoid cells at the free end.

Genus II. *Blastocaulis.* Cells are pear-shaped or globular, attached to a firm substrate by long slender stalks with a holdfast at the base. Stalks may occur singly or may arise in clusters from a common holdfast. They are not cultivated in artificial media.

Order V. *Thiobacteriales.* Characteristics given on page 362.

Family I. *Rhodobacteriaceae.* The cells are of various types, not filamentous, and contain bacteriopurpurin, with or without sulfur granules.

Genus I. *Thiocystis.* Usually 4 to 30 cells are massed into small, compact families, enveloped singly or several together in a gelatinous cyst, and capable of swarming. When the families have reached a definite size, they escape from the gelatinous cyst, the latter swelling and softening uniformly or at some particular spot. The escaped cells either pass into the swarm stage or unite into a large fused complex of families from which they separate later. Cells are light colored; single cells are almost colorless. In masses the cells show a beautiful violet or red color. The cells are frequently filled with sulfur granules.

Genus II. *Thiosphaera.* The cells are spherical-ellipsoidal, relatively large (7 to 8μ), light violet in color, and bound into loose families by a colorless gelatin. They are capable of swarming. Sulfur inclusions are relatively abundant.

Genus III. *Thiosphaerion.* The cells are spherical-elliptical, small (1.8 to 2.5μ), violet in color with delicate sulfur inclusions. They are united by means of gelatin into solid spherical families. The cells are capable of swarming.

Genus IV. *Thiocapsa.* Cell division occurs in all directions of space. The cells are spherical, with thick confluent membranes, which unite to form a structureless, gelatinous layer. The cells are of a bright rose-red color and contain sulfur granules. The cells do not swarm.

Genus V. *Thiosarcina.* Cells are nonswarming and arranged in packet-shaped families, corresponding to the genus *Sarcina.* Cells are red with sulfur granules.

Genus VI. *Lamprocystis.* Cells are ellipsoidal, dividing at first in three planes to form spherical cell masses, then later in two planes to form hollow sacks in which the cells lie embedded in a layer in the walls. Finally the membrane ruptures and the whole mass becomes net-like, much as in the algal genus *Clathrocystis.* They are usually colored intensely violet. Small sulfur granules are present. The cells are capable of swarming.

Genus VII. *Thiopedia.* Families are arranged in the form of plates. They are capable of swarming. Cells contain bacteriopurpurin and bacteriochlorin.

Genus VIII. *Thioderma.* The cells are spherical, light rose in color, and contain small, inconspicuous sulfur granules. They are united by a thin, purplish membrane.

Genus IX. *Lampropedia.* Cells are united into tetrads to form flat tubular masses and contain sulfur granules and bacteriopurpurin.

Genus X. *Amoebobacter.* The cells are connected by plasma threads. Families are amoeboid and motile. The cell families slowly change form, the cells drawing together into a heap or spreading out widely, thus bringing about a change in the shape of the whole family. In a resting condition a common gelatin is extruded; the surface becomes a firm membrane.

Genus XI. *Thiodictyon.* Cells are rod-shaped or spindle-shaped with sharply pointed ends, and united into a net. The compact mass of rods finally assumes an appearance like that of *Hydrodictyon.* The color is slightly violet.

Genus XII. *Thiothece.* Cells are spherical, in families, and enclosed in a thick, gelatinous cyst. Cells are capable of swarming and are very loosely embedded in a common gelatin. When the swarm stage supervenes the cells lie more loosely, the gelatin is swollen, and the cells swarm out singly and rather irregularly.

Genus XIII. *Thiopolycoccus.* Families are solid, nonmotile, and consist of small cells closely appressed. Multiplication takes place by breaking up of the surface into numerous, short threads and lobes, which continue to split up into smaller heaps.

Genus XIV. *Chromatium.* Cells are cylindrical-elliptical or relatively thick cylindrical. Cell contents are red and contain dark sulfur granules. Cells are somewhat variable in shape; straight, more or less bent, short ovoid, and longer forms are more cylindrical. They are motile by means of polar flagella.

Genus XV. *Rhabdomonas.* The cells are differentiated from Chromatium by the elongated, rod-shaped, or spindle-shaped cells. Cells are red and contain sulfur granules. They are motile.

Genus XVI. *Thiospirillum.* The bacteria are spiral, motile, and contain sulfur granules and bacteriopurpurin.

Genus XVII. *Rhodocapsa.* The cells are free (not united into families) and not capable of swarming (nonmotile). In mass, the organisms are cherry red. They contain sulfur granules.

Genus XVIII. *Rhodothece.* Cells are usually spherical and in pairs, and each is surrounded by a spherical or elliptical capsule. They are nonmotile. The cells are not united into families. They contain bacteriopurpurin and sulfur granules.

Genus XIX. *Rhodocystis.* Cells are rod-shaped, divide in only one plane, and are embedded in a common slimy capsule.

Genus XX. *Rhodonostoc.* Cells are spherical or short rods, in rosary-like chains, and are embedded in a common gelatinous capsule.

Genus XXI. *Rhodorrhagus.* Cells are spherical, nonmotile, free, and not united into families.

Genus XXII. *Rhodobacterium.* Cells are rod-shaped, nonmotile, and not united in families.

Genus XXIII. *Rhodobacillus.* Cells are rod-shaped, solitary, and usually motile.

Genus XXIV. *Rhodovibrio.* Cells are short, comma-shaped, free, and actively motile by means of a polar flagellum.

Genus XXV. *Rhodospirillum.* Cells are spiral and actively motile by means of polar flagella.

Family II. *Beggiatoaceae.* Bacteria are filamentous, and usually show an oscillating motion similar to *Oscillatoria.* Cells contain sulfur granules. Spore formation and conidia are unknown.

Genus I. *Thiothrix.* Filaments are nonmotile, segmented, with a definite differentiation into base and tip, attached, and usually filled with sulfur granules. The threads produce rod-shaped conidia at the ends. These conidia are motile, exhibit a slow, creeping movement, and attach themselves and develop into threads. The habitat is in hot sulfur springs.

Genus II. *Beggiatoa.* The threads are sheathless, formed of flat, discoidal cells, and not attached. Multiplication occurs by transverse splitting of the threads. The threads show an undulatory creeping motion. Cells contain granules of sulfur.

Genus III. *Thioploca.* Filaments are *Beggiatoa*-like, with numerous sulfur granules. They are motile. Filaments lie parallel in considerable numbers or are united in bundles enclosed in a colorless layer of gelatin.

Family III. *Achromatiaceae.* Organisms are unicellular, large, and motile. Cells contain granules of sulfur but no bacteriopurpurin.

Genus I. *Achromatium.* Cells are large and nearly spherical to ellipsoidal. Cells are closely packed with large granules, at first interpreted as sulfur but later as calcium oxalate. When granules are dissolved, the cells show a coarse structure. Cells are motile. Cell division resembles the constriction of flagellates rather than the fission characteristics of bacteria.

Genus II. *Thiophysa.* Cells are spherical and the cell membrane is loaded with sulfur granules. The protoplasmic layer surrounds a large central vacuole. The oxalate is contained in the vacuole. Cell nucleus is not recognized. Flagella are lacking. Cells elongate before division and divide into biscuit-shaped cells. In the presence of an excess of oxygen the sulfur drops disappear and only the oxalate remains. In the absence of oxygen and in the presence of hydrogen sulfide, the oxalate disappears and sulfur drops fill the cell.

Genus III. *Thiospira.* Cells are colorless, motile, slightly bent, somewhat pointed at the ends, contain sulfur granules and a small number of flagella at the ends.

Genus IV. *Hillhousia.* Cells are very large and motile by means of peritrichous flagella. Cells are packed with large globules of oily, amorphous sulfur.

Order VI. *Myxobacteriales.* Characteristics given on page 362.

Family I. *Archangiaceae.* In the organisms belonging to this family the swarm (pseudoplasmodium) produces irregular swollen or twisted fruiting bodies, or develops columnar or finger-like growths usually without a definitely differentiated membrane.

Genus I. *Archangium.* The mass of shortened rods embedded in slime form a pad-shaped or more rounded, superficially swollen or tuberous fruiting body, even with horny divisions. The fruiting body has no membrane. In the interior can be seen a mass resembling coiled intestines. The windings of this coil may be uniform or irregularly jointed, free, or stuck together; the ends may be extended and horny. Instead of a membrane there may be loosely enveloping slime.

Genus II. *Stelangium.* Fruiting bodies are columnar or finger-like, sometimes forked, without definite stalk, and standing upright on the substrate.

Family II. *Sorangiaceae.* The shortened rods of the fruiting body lie in angular, usually relatively small cysts of definite polygonal shape. Often many of

these cysts are surrounded by a common membrane. The primary cyst may be differentiated from the angular or secondary cysts. No stalked forms are known.

Genus I. *Sorangium* The cysts are united into rounded fruiting bodies.

Family III. *Polyangiaceae.* In the fruiting bodies the more or less shortened rods lie in rounded cysts of definite form. The well-defined wall is composed of hardened slime and is yellow, red, or brownish. The cysts may be united by a definitely visible slime membrane, the remnant of the vegetative slime, or they may be tightly appressed and cemented by the scarcely visible remnants of the slime, or they may develop singly or in numbers on a stalk. In the more highly developed forms the stalk branches and carries the cysts at the tips of the branches.

Genus I. *Polyangium.* Cysts are rounded or coiled, surrounded by a well-developed membrane and either free or embedded in a second slimy layer.

Genus II. *Synangium.* Cysts are provided with an apical point, and united more or less completely to rosette-shaped, hemispherical, or spherical fruiting bodies.

Genus III. *Melittangium.* Cysts are brownish orange red, on short white stalk, like a mushroom. It has the appearance of a white-stalked *Boletus.* The rods inside stand at right angles to the membrane. Upon germination, the covering membrane is left colorless and with an appearance of honeycomb.

Genus IV. *Podangium.* Cysts are chestnut brown or red brown, and single on a more or less definite white stalk.

Genus V. *Chondromyces.* Cysts are compactly grouped at the end of a colored stalk (cystophore). Cystophore may be simple or branched.

Family IV. *Myxococcaceae.* The rods become short when cysts are formed, and develop into spherical spores. Upon germination they elongate to rods without rupturing the membrane.

Genus I. *Myxococcus.* Spherical spores are present in conical, or spherical, or occasionally ovoid, upright fruiting bodies, united by a loose more or less mobile slime.

Genus II. *Chondrococcus.* Spores are embedded in a viscous slime, which hardens. Fruiting bodies are divided by joints or constrictions, often branched, and usually relatively small.

Genus III. *Angiococcus.* Fruiting body consists of numerous round (disk-shaped) cysts. Cyst wall is thin with spores within.

Order VII. *Spirochaetales.* Characteristics given on page 363.

Family I. *Spirochaetaceae.* Characters are the same as for the order.

Genus I. *Spirochaeta.* The organisms are nonparasitic with flexible, undulating body and with or without flagelliform tapering ends. They are common in sewage and foul waters.

Genus I*a*. *Cytophaga.* Rods are long, flexuous, with pointed ends and show metachromatic granules. They are incapable of using carbonaceous materials as food, except cellulose, which is hydrolyzed. Growth in ordinary culture media is feeble.

Genus II. *Saprospira.* The cells are nonparasitic and similar to *Cristispira,* but without the flattened ridge or "crista," which is, if present, here replaced by a straight columella or thickening of the periplast.

Genus III. *Cristispira.* These are giant forms with undulating body and peculiar flattened ridge, which is erroneously called an undulating membrane and which runs the length of the body. They are parasitic in molluscs.

Genus IV. *Borrelia.* The forms are small, parasitic, spiral, and flexible with terminal filaments. The spirals are large, wavy, and three to five in number.

Genus V. *Treponema.* The organisms have an undulating or rigid spirilliform body, without crista or columella. Flagelliform tapering ends may or may not be present. They are parasitic and frequently pathogenic.

Genus VI. *Leptospira.* The organisms are sharply twisted cylinders with flagelliform tapering ends, one extremity being sharply curved into a hook. They are parasitic forms.

It may be seen that the classification of bacteria is an exceedingly difficult task. The work becomes increasingly more difficult as more and more new species are discovered and studied. Some organisms that were at one time placed in certain genera are now transferred to other genera or placed in new ones. This is to be expected when the difficulties encountered in studying such minute organisms are considered. It is highly probable that no single classification will ever be completely acceptable to all bacteriologists but the one given here is undoubtedly the best of any that have ever been proposed and it is in general use in this country.

For an excellent discussion on bacterial classification see the report of Stanier and van Niel (1941).

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CHAPTER XVII

DISSOCIATION OF BACTERIA

During the early years of bacteriology most bacteriologists were pleomorphists, believing that a bacterial species could exist in more than one cell form. Some years later this concept was altered in favor of the monomorphistic hypothesis or fixity of bacterial cell form. Forms that departed more or less widely from the normal types were usually dismissed as being either involution forms, degenerate cells, or different species present as contaminants. At the present time sufficient evidence has accumulated to support the original pleomorphic hypothesis or variability in the morphological characteristics of an organism.

Considerable literature has accumulated during the past twenty-five years concerning these aberrant forms and the results have proved, without a doubt, that the concept of monomorphism can no longer be held. The question of the instability of bacterial species is of tremendous importance to all branches of bacteriology. The work of the systematic bacteriologist (classification of bacteria) becomes more difficult than was at first supposed because an organism may show variations in physiological reactions as well as in morphological characteristics. The phenomenon concerns also the question of infection, immunity, virulence, and many other phases of bacteriology.

Several terms have been used in connection with the phenomenon of bacterial variation. These terms have been used more or less loosely and, for this reason, are the cause of considerable confusion. It is, perhaps, appropriate at this time to define them according to their true meaning.

Mutation.—This term was first defined by De Vries as a sudden variation, the daughter cells differing from the mother cells in some well-marked character or characters, as distinguished from a gradual variation in which the new characters become developed only in the course of many generations. On the other hand, Dobell believes that mutation includes any permanent change transferred to the daughter cells regardless of whether it is a sudden or a gradual change. The former definition is the preferred one.

Involution.—The term is defined as a retrograde development or the appearance of degenerate cells. The presence of certain harmful substances incorporated in culture media will, when inoculated with some

species, cause the appearance of forms which depart widely from the normal and which are sufficiently characteristic to be of diagnostic importance.

Variation.—Variation is defined as a divergence in the morphological or physiological characters (usually both) of a species from those observed in the original cells of the culture. The term is usually used synonymously with dissociation but is often regarded as opposed to heredity.

Life Cycles in Bacteria.—Some investigators believe that bacteria pass through a complex life cycle similar to that displayed by protozoa. It is true that bacteria do show variations in morphology and physiology but these are the result of changes in the environment and correlate with the phases of growth. In other words, all forms of the cycle are not present at the same time. As the environment changes, different morphological or physiological characters (or both) develop. Lewis (1932, 1933, 1937, 1938) in a series of studies on *Bacillus mycoides*, *Azotobacter chroococcum*, *A. beijerinckii*, *Rhizobium meliloti*, and *R. trifolii* concluded that there was no evidence to support the concept that variant forms that appear in a culture represent phases of a complex pleomorphic life history through which an organism must pass in a cyclogenic method of development. It is, therefore, quite unlikely that bacteria pass through an orderly life cycle.

Bacterial Instability.—The instability of bacterial species manifests itself in various ways. Coccus forms may under some conditions change to rods and rod forms may change to cocci; motility may disappear only to return again; cells that take an even and uniform stain when young may show a granular appearance when old; physiological reactions such as the fermentation of carbohydrates may become modified; sporulating organisms may lose their ability to produce spores (asporogenous); capsulated organisms may lose their power to secrete a capsule, and species that normally do not produce capsules may be made to do so by modifying the composition of the culture medium or the temperature of incubation; the antigenic response shows marked variation; some species in culture agglutinate spontaneously and others lose their ability to agglutinate; virulent cultures may lose their power to produce disease and harmful avirulent strains may become virulent. These are only a few examples of the types of variations that have been recorded in the literature. Thompson (1935) grouped bacterial variations as shown in Table 42.

Variations in Cell Size with Age.—It is well known that young cells are, in general, larger than old organisms. As a freshly inoculated culture ages, the cells become progressively larger and larger until a maximum is reached, after which the reverse effect takes place. Henrici (1928) measured the lengths of cells of *Bacillus megatherium* growing in a micro-

TABLE 42.—TYPES OF BACTERIAL VARIATIONS

Group	Character affected	Variations observed
1	Size of cells..............	Minute forms (filterable?) Giant cells
2	Cell morphology...........	Coccoid forms in bacillary cultures Bacillary forms in cultures of cocci Filamentous forms Club forms Branched forms Bizarre cells of various shapes Ameboid forms—"symplasms"
3	Staining properties	Gram-negative forms in Gram-positive species Nonacid-fast forms in acid-fast species Irregular staining
4	Spore formation	Nonsporing variants in spore-forming species Variations in method of germination of spores
5	Motility . . .	Loss of flagella in motile species
6	Capsule. . . .	Increase or decrease in size of capsule Complete loss of capsule (?)
7	Shape ar [illegible] of colonies on [illegible] . .	Smooth (*S*), rough (*R*), mucoid (*M*), dwarf (*D*) or gonidial (*G*) Irregular, spreading (*H*) or raised, discrete (*O*) Pigmented or nonpigmented colonies Secondary papillae on colony surface Moth-eaten colonies Opaque or translucent colonies Creamy or sticky colonies
8	Type of growth in broth	Diffusely turbid or granular sedimenting Pellicle or no pellicle Slimy sediment or diffuse clouding
9	Nutritional requirements	"Growth-promoting" substances necessary or not Serum or other complex body substances necessary or not Changes in O_2 requirements
10	Fermentation of carbohydrates...... . .	Loss of fermenting power typical for species. Acquisition of a fermenting power not typical for species
11	Proteolysis	Loss of proteolytic power
12	Hemolysin production	Loss or gain of power to produce hemolysin
13	Toxin production	Loss or gain of power to produce toxin
14	Pigment production	Increase or decrease of pigment formation
15	Virulence	Decrease or increase of virulence in general Decrease or increase of virulence for a particular animal species Complete loss of virulence
16	Resistance to harmful influences.	Variations in resistance to heat, chemicals, autolysins, antibodies, bacteriophage
17	Antigenic components.......	Presence or absence of flagellar antigen [illegible] group- or species-specific [illegible]ohydrate (*S* antigen) present or absent Changes in *S* antigen

colony after gradually increasing periods of incubation. The results are shown in Fig. 142. The corresponding measurements of cell size, together with the number of cells produced in each time period, are recorded in Table 43 (see also page 225).

Interrelationships of Variations.—It is well known that many variations are interrelated. The loss of flagella results not only in the appear-

ance of a nonmotile variant but affects the antigenic response and colonial morphology as well. Weil and Felix (1917) applied the names "hauch" (H) and "ohne hauch" (O) to two types of Proteus X_{19} colonies which appeared on agar plates. The H colonies were composed of motile organisms, whereas the O colonies showed only nonflagellated organisms.

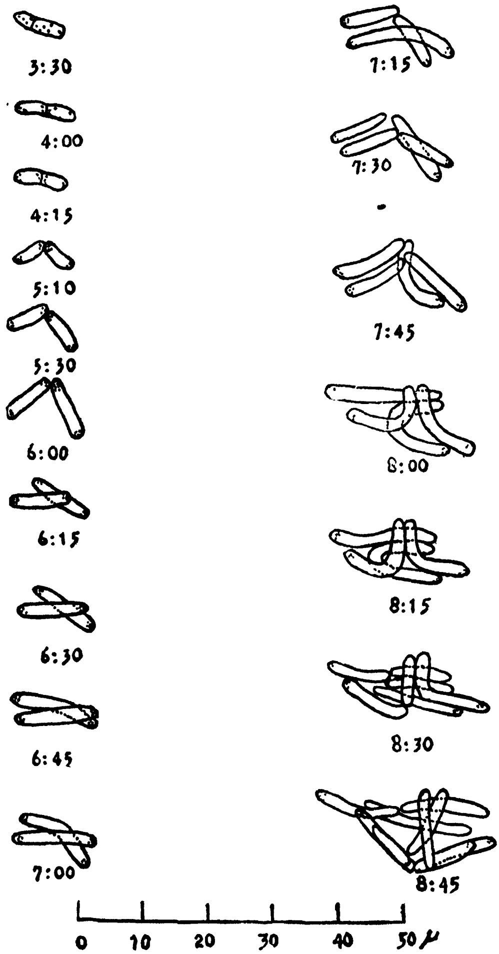

FIG. 142.—Morphologic variation in a growing microcolony of *Bacillus megatherium*. (*From Henrici, Morphologic Variation and the Rate of Growth of Bacteria, Charles C. Thomas, Springfield, Ill.*)

The flagella are capable of eliciting an antigenic response different from that of the bacterial bodies. The motile cells (H) produce a flocculent agglutination whereas the nonmotile organisms (O) give a granular agglutination. The nonmotile forms produce colonies that are discrete and domed as opposed to the flat, confluent growth displayed by the

TABLE 43.—RATE OF GROWTH AND CELL SIZE OF *Bacillus megatherium*

Minutes of growth	Number of cells in colony	Average length of cells, μ	Sum of lengths of cells, μ
0	2	3.7	7.5
30	2	4.7	9.4
45	2	4.7	9.4
100	2	5.6	11.2
120	2	6.6	13.1
150	2	8.8	17.5
165	2	9.2	18.5
180	2	10.6	21.3
195	2	13.1	26.3
210	2	12.8	25.6
225	3	12.1	36.3
240	4	9.8	39.4
255	4	11 6	46.3
270	4	17.3	69.4
285	4	16.9	67.5
300	7	11.0	76.9
315	9	12.1	109.4

motile cells. The presence or absence of flagella affects the results in groups 5, 7, and 17 of Table 42.

COLONY FORMS

Several types of colony forms have been recognized in bacterial cultures. These have been designated as mucoid (*M*), smooth (*S*), intermediate (*SR*), rough (*R*), dwarf (*D*), and gonidial (*G*) colony forms or phases.

S and R Forms.—Griffith (1923) noted two types of colonies when a pure culture of the pneumococcus was streaked over the surface of a solid medium. One type of colony was dull and granular; the other was shiny and smooth. He designated the former type as the rough or *R* variant and the latter as the smooth or *S* variant. The *S* forms possessed distinct capsules whereas the *R* variants were noncapsulated. Since then *R* and *S* variants have been shown to be of general occurrence.

Dawson (1934) succeeded in isolating a rough variant from the pneumococcus, which was different from the type reported by Griffith. He showed that what Griffith had called the rough phase was in reality the smooth variant, whereas the phase previously considered the smooth variant became the mucoid or *M* variant. The mucoid phase has been

recognized in a number of organisms particularly in those members of the colon-typhoid-dysentery group.

A rough colony answers to the following description: The margin is very [illegible]. the surface is very flat, uneven, and granular; the organisms produce a granular sediment in broth and clump spontaneously in physiological salt solution.

A smooth colony may be described as follows: The margin is round and even; the surface is convex, smooth, and glistening; the organisms

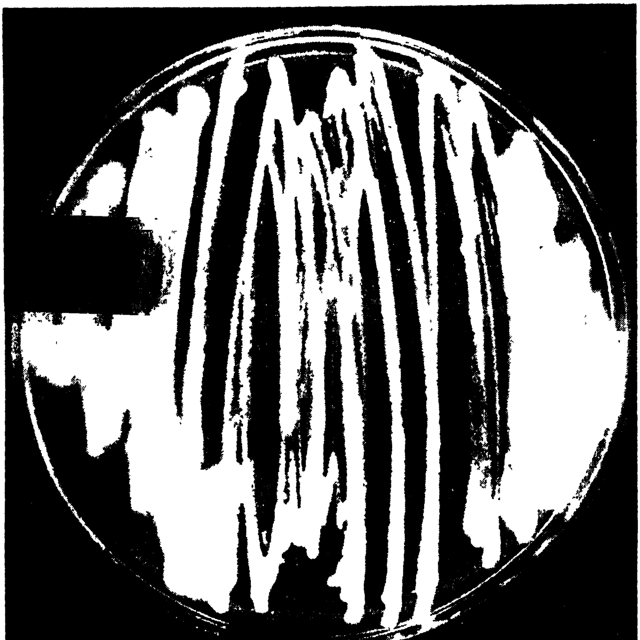

FIG. 143.—Mucoid culture of *Escherichia coli* from rat feces. Variant obtained by incubating the plate at 15°C. for four days. (*Plate prepared by H. R. Morgan.*)

grow as a uniform turbidity in broth and produce a stable suspension in physiological salt solution.

SR Forms.—An *SR* colony is not necessarily one that is intermediate between a smooth and a rough type. It means that the colony has an appearance that is intermediate between the *S* and the *R* forms. It arises from one form and proceeds into the other.

M Forms.—An *M* culture form is characterized by the appearance of colonies having a pronounced moist, glistening, mucoid consistency. The colonies show a strong tendency to run together (Fig. 143). The cells are often capsulated. Both these characters have been observed in species in which such attributes were not believed to be present.

D Forms.—Occasionally a pure culture of an organism, when streaked over the surface of a solid medium, may show the presence of two varieties of colonies designated as large and small colony forms. When separated into pure cultures, the two forms usually maintain their characteristic difference in size for many generations. Pure cultures of each type, after many generations, may show the presence of some colonies of the other type. The small colony type is referred to as the dwarf or *D* form. These colonies are very small, sometimes scarcely visible, and measure about 1 mm. or less in diameter (Fig. 144). The organisms in both the large and the small colonies appear to be the same in morphology and physiology, differing only in colony size.

A B C D

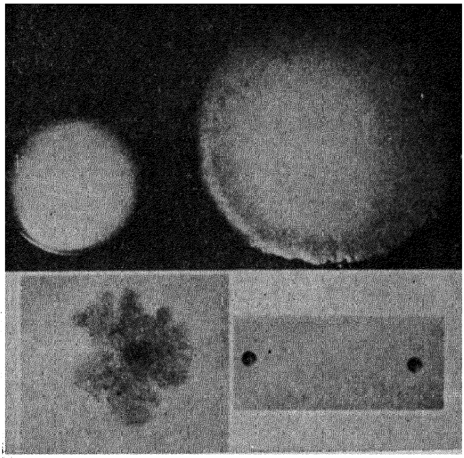

Fig. 144.—The four main colony types of *Corynebacterium diphtheriae*. *A*, smooth (*S*) colony grown 48 hr. at 37°C. and magnified 12 times; *B*, intermediate (*SR*) colony grown 48 hr. at 37°C. and magnified 12 times. The two colonies were found growing side by side; *C*, rough (*R*) colony grown 48 hr. at 37°C. and magnified 18.8 times; *D*, dwarf (*D*) colony grown 48 hr. at 37°C. and magnified 18.8 times. (*After Morton.*)

G Forms.—Sometimes the *D* forms have been confused with another minute colonial type designated as the *G* or gonidial form. The *G* colonies represent growth from filterable elements of the bacteria. In this respect the *G* form differs from all the other variants that have been observed.

There appears to be considerable disagreement among bacteriologists on the question of the presence of filterable forms of bacterial species. Some have reported filterable stages in certain species; others have been unable to verify the results.

The filterable forms in cultures yielding positive results are related to the gonidial bodies present in cultures under certain environmental conditions. In the absence of the *G* forms negative filtration results will follow. Certain conditions must be fulfilled before it can be concluded

that one is dealing with filterable forms rather than with the normal cell types that have passed through large pores in the filters. As Hadley stated,

It is probably only under exceptionally favorable conditions that genuine filterable forms can be demonstrated easily and with any degree of regularity. The original state of the culture material is the primary requisite. Because of this it would appear more important than is indicated by many recent works on filtration to possess, at the beginning of the experiments, some assurance that bacterial elements, whose morphology and size suggest their possible filtrability, are present before filtration in the material subjected to the test. Aside from this the next most important requisite is to utilize such mediums or conditions of cultivation as will ensure the upgrowth of the filterable elements, presumably the gonidia, regarding whose frequent dormancy there can be no question.

M to S to R Change.—The smooth phase has been observed most frequently in the majority of bacterial species. This is followed by the rough phase. The mucoid variant has been observed the least of all.

Some believe that the different culture phases are, in most cases at least, easily convertible one into the other. There appears to be no evidence that any culture phase is stable in the true sense of the word, although it is true that some phases have not been reversed by the employment of in vitro methods. From the small number of observations at hand it appears that the passage of a culture from one phase to another is not a haphazard transformation but follows a definite sequence of changes. The transition from *S* to *M* occurs more frequently than the change from *R* to *M*. The change from *S* to *M* is less commonly observed than the change from *M* to *S*. In arranging all the transformations reported in the literature, Hadley (1937) found that the sequence of *M* to *S* to *R* occurred more frequently than any of the others and appeared to be the most securely placed of all.

Secondary Colonies.—It has long been known that, under certain conditions, old or senescent colonies of many species of bacteria may again resume growth. This is generally referred to as the secondary growth phase. The secondary growth phase may be of short duration, resulting in the formation of minute protuberances or papillae, or it may be more prolonged, resulting in the formation of well-developed daughter colonies. Lewis (1933), in his studies on the dissociation of *Bacillus mycoides*, explained the appearance of secondary colonies by stating:

The secondary phase of growth is due to depletion of preferred nutrients and subsequent utilization of unused substances by certain cells. Ability to attack unused nutrients is acquired through variation due to the specific stimulus exerted by the substance concerned. The substances found suitable for pro-

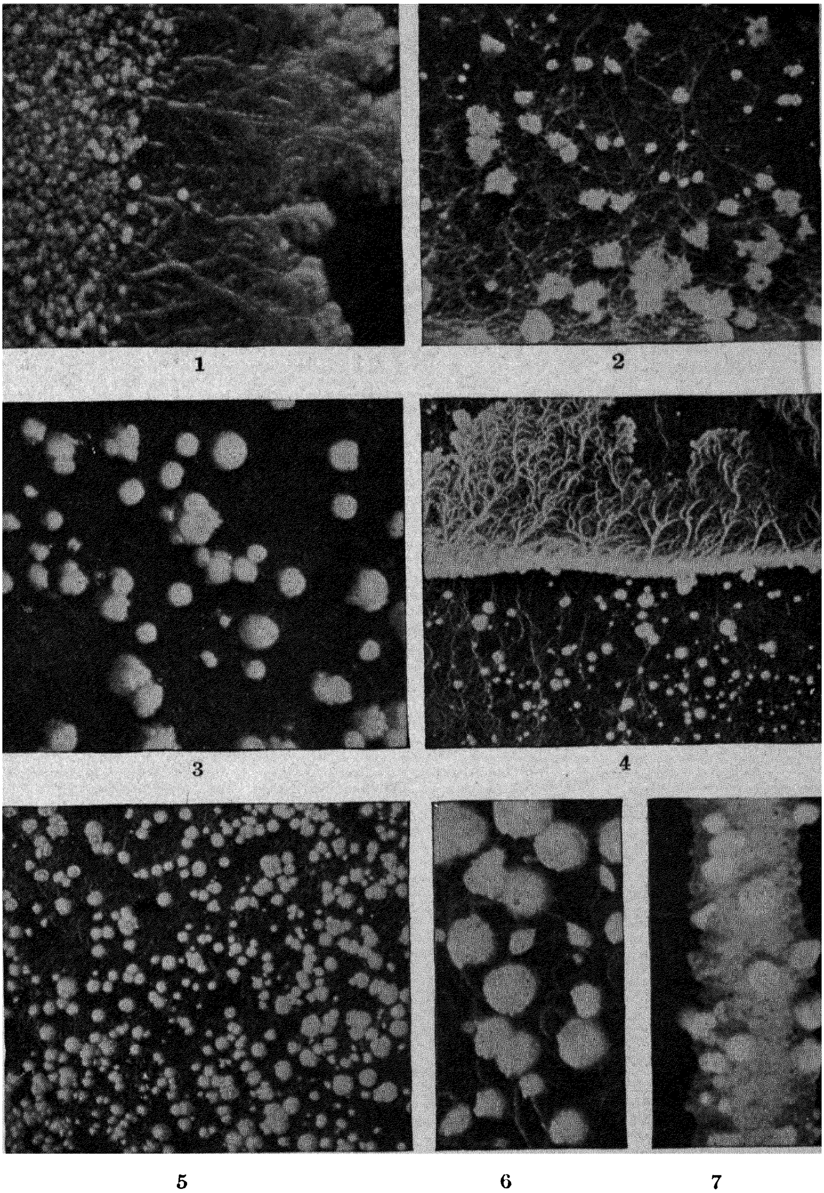

FIG. 145.—Secondary colonies of *Bacillus mycoides*. 1, portion of a giant colony of strain *B* on agar prepared from 14-day gelatin culture and photographed on the tenth day. 2, colonies of strain 421 at end of 14 days on agar containing 1 per cent peptone. 3, colonies of strain *A*, 60-day gelatin culture, on agar containing 0.5 per cent peptone and 0.3 per cent beef extract and photographed on the tenth day. 4, strain A.T.C. at end of 14 days on nutrient agar plus 2 per cent sucrose. Mixture of original and sucrose variant types. 5, strain A.T.C. on nutrient agar plus 2 per cent sucrose and photographed at the end of 21 days. 6, portion of same colony shown in (5). The original threads show only faintly at this age. 7, strain *A* at end of 30 days prepared as described under (3). The colonies are shown natural size in (4) and (5). All others are magnified 3.5 times. (*After Lewis.*)

moting secondary growth were sucrose and protein fractions of unknown identity contained in digested meat, casein, or gelatin.

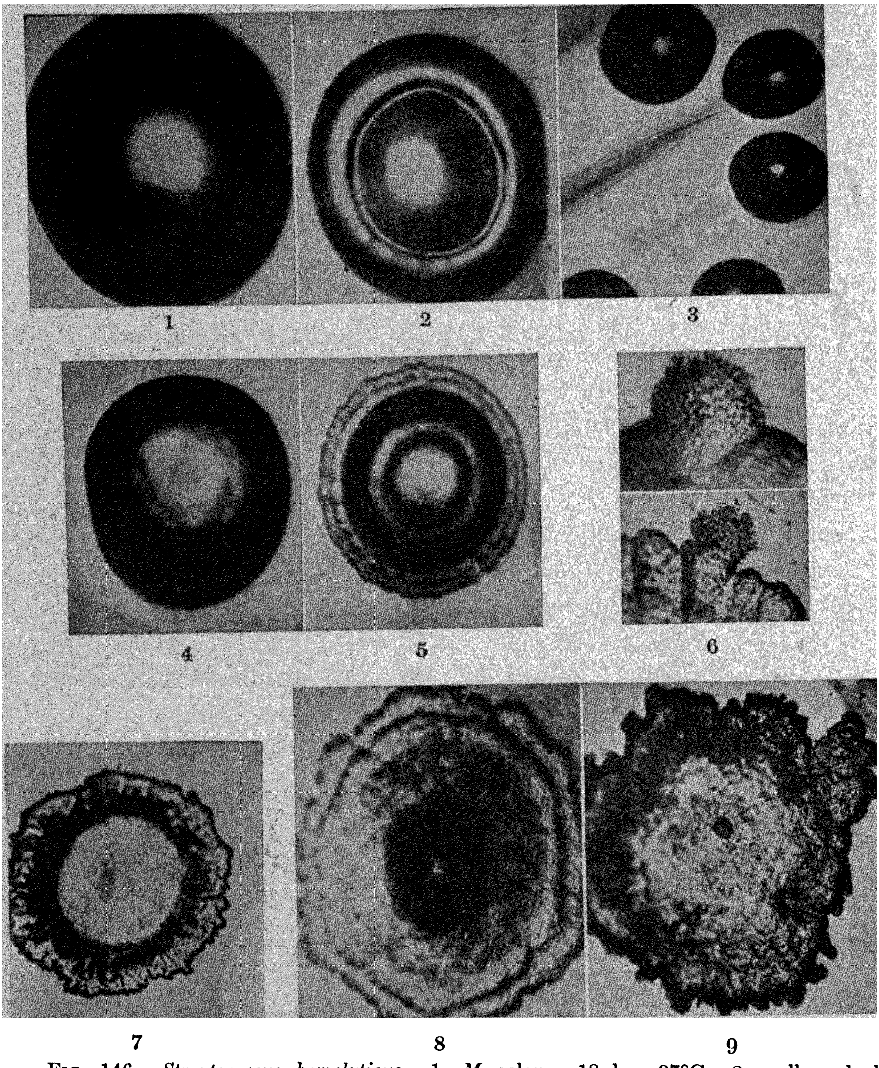

FIG. 146.—*Streptococcus hemolyticus.* 1, *M* colony, 18 hr., 37°C.; 2, well-marked *S* → *M* variation, 18 hrs., 37°C.; 3, matt colonies, 18 hrs., 37°C.; 4, *S* colony, two days, 37°C.; 5, early *SR* colony, four days, 37°C.; 6, *R* variation at margins of *SR* colonies, six days, 37°C.; 7, *SR* colony approaching pure *R*, three days, 37°C.; 8, *R* colony (possibly some *SR* elements at center), three days, 37°C.; 9, *R* colony, eight days, 37°C. (*After Dawson, Hobby, and Olmstead.*)

The subraces established from secondary colonies show enhanced capacity for utilization of the compound to which variation occurred and do not again produce secondary colonies in its presence. The variants are relatively stable.

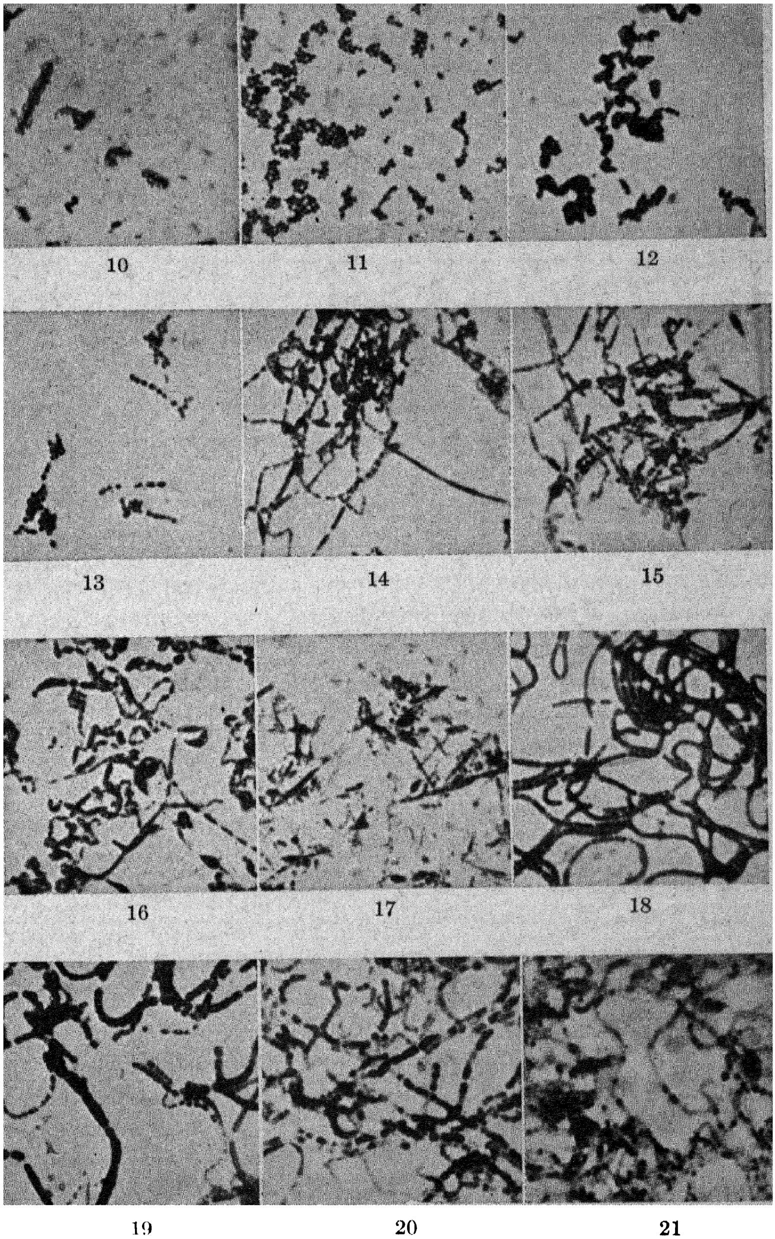

Fig. 147.—*Streptococcus hemolyticus.* 10, *M* organisms from 3-hr. colony, 37°C., Hiss capsule stain; 11, *M* organisms from 15-hr. colony, 37°C., Gram stain; 12, matt organisms from 15-hr. colony, 37°C., Gram stain; 13, *S* organisms from 72-hr. colony, 37°C., Gram stain; 14–17, successive stages in $S \rightarrow R$ variation, 37°C., Gram stain; 18, *R* organisms from 24-hr. colony, 37°C., Gram stain; 19–21, successive stages in $R \rightarrow S$ variation, 37°C., Gram stain. (*After Dawson, Hobby, and Olmstead.*)

Illustrations of secondary colonies of *B. mycoides* are shown in Fig. 145.

RELATION OF DISSOCIATION TO CELL MORPHOLOGY

Earlier studies have shown that bacterial cells increase in length as they pass from the smooth to the rough phase. Tangled masses of filaments were usually observed among cultures of the rough variants. Recently Stevens (1935) noted increasing cell size in *Clostridium perfringens* in passing from the mucoid to the smooth to the rough type. Dawson (1934) and others observed the same morphological picture in cultures of the pneumococcus. More recently Dawson, Hobby, and Olmstead (1938), in a study of the variants of several types of streptococci, came to a similar conclusion (Figs. 146 and 147).

RELATION OF COLONY FORM TO OTHER ATTRIBUTES

In earlier studies and before the mucoid phase was recognized as an entity, it was shown that the smooth variant carried greater virulence than the rough variant (which was usually avirulent). Results that have been reported in the literature during the past fifteen years have shown that the above conclusions are essentially correct. Rough cultures, as a rule, lack both virulence and toxigenicity. Among those bacterial species in which the mucoid phase has been commonly recognized this variant has been shown to carry maximum virulence and toxigenicity. Exceptions to this have been noted. Hemolysis (dissolution of red blood corpuscles by toxins secreted by some bacterial species) does not appear to be correlated with any of the variants.

Hadley (1927), in a review of the literature, showed that each culture phase was closely correlated with other morphological, cultural, and physiological characteristics, such as presence of capsules, morphology of the cell, immunological reactions, antigenic structure, virulence, motility, sensitiveness to bacteriophage, resistance to phagocytosis, etc. Since that time reports have appeared suggesting that many exceptions exist, that these more recent observations support the general view that each characteristic of a species is subject to independent transmissibility. In a later communication Hadley (1937) summarized these observations by stating,

> . . . although certain attributes of a bacterial species may be able to vary independently of the culture phase, these instances are so infrequent as hardly to affect the broad generalization that each culture phase, when existing in a relatively pure state, is closely related to a certain group of characters; and that, when one phase has become fully transformed into another, some of these attributes are lost while new ones are gained.

The characters that have been found more frequently to correlate with phase include cell morphology, cell grouping, motility, possession of specific carbohydrates, tendency to saprophytic existence, antigenic structure (including serological and immunological features), toxigenicity, and virulence. The characters that have been found not to correlate clearly with phase are chromogenesis, hemolysis, fermentations, and other physiological reactions.

FACTORS INCITING VARIATIONS

Variations may be classed as (1) nonhereditary and (2) hereditary. The former group includes those variations which result from changes in the environmental conditions. The latter group includes those variations which may be permanent or continue for a limited number of generations.

Most of the variations that have been observed are probably nonhereditary in character and result from changes in the environment. Variants may be easily observed by long-continued growth of an organism in a culture without transfer. Examination of smears prepared at definite intervals reveals the presence of forms that depart widely from the so-called normal type. The transfer of the variants from an old culture to a fresh tube of the same medium, followed by incubation, results in a reappearance of the "normal" cell form.

Revis (1911) noted that when cultures of *Escherichia coli* were treated with malachite green, the organisms lost their ability to produce gas from fermentation of lactose, glucose, dulcitol, and mannitol. Acid formation, however, was not destroyed. The new physiological condition of the organism was found to be quite permanent and all attempts to reproduce the power of gas formation failed. In other respects the organisms appeared to be identical with those in the original cultures.

Penfold (1911) streaked a culture of *E. coli* over the surface of agar containing small amounts of sodium monochloracetate. He obtained two types of colonies: (1) a normal type and (2) a dissociant type, which failed to produce gas in glucose, levulose, mannose, galactose, arabinose, xylose, lactose, dextrin, and salicin, but still able to produce gas from the alcohols dulcitol, mannitol, and sorbitol. The variant retained the new characteristics for several generations. In a later communication Penfold (1913) substituted monochlorhydrin for the sodium monochloracetate and found that *E. coli* threw off variants similar to those produced on the former medium.

Phenol agar (0.1 per cent) was found by Braun and Schaeffer (1919) to change the *H* or flagellar variant of *E. coli* to the *O* or nonmotile form. This change was only transient, the *H* form reappearing on transfer of the organism to the usual laboratory media.

The addition of chemicals (germicides) and dyes (bacteriostatic agents) to culture media frequently results in the appearance of variants that are sufficiently characteristic to be used in identifying some species.

Three procedures are usually followed in securing pure cultures of variants: (1) growth of the organisms under environmental conditions inducing them to dissociate, (2) the use of selective culture media encouraging the appearance of variants, (3) macroscopic selection of the characteristic colonies appearing on the surface of solid media.

The Cause of Variations.—The cause of variations is not definitely known. Some maintain that variations represent stages in an orderly life cycle of the organisms. Others believe that the changes are of a hereditary nature, involving chromosomes and sex cells leading to the evolution of new forms and species. The presence of chromosomes and sex cells in the true bacteria is still doubted by the majority of bacteriologists. The cause of variations must await the outcome of further work in this field.

DISSOCIATION AND CLASSIFICATION OF BACTERIAL SPECIES

The scheme that has been employed for the classification of bacteria is based on the monomorphic concept of bacterial characteristics. This means that each species possesses only one phase. It is now definitely established that bacteria are pleomorphic, that they possess more than one cultural phase. If bacteria are classifiable, the question that immediately arises is which phase may be considered to be the normal form. Some believe that, at the present time, bacteria are not classifiable and will not be until bacteriologists learn more about what constitutes a bacterial species.

For further reading consult the excellent articles by Bisset (1938), Haddow (1938), Hadley (1939), McGaughey (1933), Mellon (1942), Morgan and Beckwith (1939), Parr and Robbins (1942), Shinn (1939), Winslow and Walker (1939), and Youmans and Delves (1942).

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CHAPTER XVIII

ASSOCIATIONS OF BACTERIA

Organisms are rarely, if ever, found growing as pure species in their natural habitat. Mixed cultures of two or more species are the general rule. Because of this fact it is sometimes erroneous to conclude from laboratory findings the exact changes organisms produce in their natural environment.

Simple mixtures of two species may exist in which the organisms produce no effect on each other, but this is rarely true. Associations may exist between different species of bacteria and bacteria with other classes of organisms, such as algae, protozoa, molds, and yeasts.

Symbiosis.—In many cases growth and multiplication are more vigorous in friendly associations than with either species existing alone. Such a phenomenon is spoken of as symbiosis. This term may be defined as the living together of two or more species of organisms in friendly association for mutual benefit.

Certain soil bacteria belonging to the genus *Rhizobium* are found in tumors or nodules produced on the roots of plants belonging chiefly to the family Leguminosae. These organisms utilize free atmospheric nitrogen and build it up into organic compounds. The plants are furnished available nitrogen by the bacteria and the bacteria derive their nutrients from the plant sap. A perfect symbiotic relationship exists (see page 496).

Many examples of symbiosis reported in the literature are misnomers in that only one of the organisms in the association is apparently benefited. The favorable influence of an aerobe on the growth of an anaerobe may be mentioned. The aerobe reduces the oxygen tension and creates an environment suitable for the growth of the anaerobe. The anaerobe is benefited by the association while the aerobe is either not affected or is harmed. This should be regarded as an example of commensalism or of antibiosis rather than of symbiosis. True examples of bacteria growing in symbiosis with other species of bacteria where both are benefited by the association are rare in nature.

Commensalism.—An organism may be unable to grow in the presence of a certain substrate. If, however, another organism is present, capable of attacking the food material with the production of a compound or compounds utilizable by the first organism, growth will occur. Such an

association is spoken of as commensalism. The term commensalism may be defined as the living together of two species, one of which is benefited by the association while the other is apparently neither benefited nor harmed.

Waksman and Lomanitz (1925) showed that *Bacillus cereus* developed very rapidly in a synthetic medium containing casein as the only source of nitrogen. The casein was vigorously hydrolyzed with the production of amino acids and other split products. The hydrolysis took place more rapidly in the absence of a fermentable carbohydrate. *Pseudomonas fluorescens*, on the other hand, was unable to attack and utilize the casein either for structure or for energy. However, the organism utilized amino acids with ease (Fig. 148).

$$\begin{tikzcd}
\text{Casein} \arrow[r, "B.\ cereus"] & \text{Amino acids} \arrow[d, "P.\ fluorescens"'] \\
& \begin{array}{l} N \text{ for structure} \\ C \text{ for energy} \end{array}
\end{tikzcd}$$

FIG. 148.—Action of *B. cereus* and *P. fluorescens* on casein and its split products.

B. cereus is a typical proteolytic organism capable of degrading native proteins to compounds having smaller molecular weights. *P. fluorescens* is nonproteolytic but can utilize free amino acids as a source of nitrogen for structure and carbon for energy.

When both organisms were inoculated into the above medium, the changes that took place on the casein molecule were different from those produced by each organism acting separately. *B. cereus* first attacked the casein with the liberation of amino acids and other compounds. *P. fluorescens* decomposed the amino acids as they were formed. The result was that the culture soon showed more cells of *P. fluorescens* than of *B. cereus*. *P. fluorescens* was definitely benefited by the association while *B. cereus* was probably neither benefited nor harmed.

Gale (1940) reported that *E. coli* was capable of decarboxylating arginine to agmatine and ornithine to putrescine but was unable to hydrolyze arginine to ornithine. On the other hand, *Streptococcus faecalis* was capable of hydrolyzing arginine to ornithine but was incapable of decarboxylating arginine to agmatine or ornithine to putrescine (Fig. 149).

$$\begin{tikzcd}
\text{Arginine} \arrow[r, "E.\ coli"] \arrow[d, "S.\ faecalis"'] & \text{Agmatine} \\
\text{Ornithine} \arrow[r, "E.\ coli"] & \text{Putrescine}
\end{tikzcd}$$

FIG. 149.—Action of *E. coli* and *S. faecalis* on arginine and ornithine.

In a medium containing arginine neither *E. coli* nor *S. faecalis* acting separately could produce putrescine. However, when both organisms were inoculated into the same medium, *S. faecalis* produced ornithine, which was then acted upon by *E. coli* to give putrescine. *E. coli* was benefited by the association while *S. faecalis* probably was not affected.

Another example of commensalism that is frequently employed in the laboratory is the cultivation of an anaerobe in the presence of a facultative aerobic organism. An appropriate solid medium is poured into a Petri dish and the agar allowed to become firm. The anaerobic organism is streaked over the surface of one-half of the plate and the facultative aerobe is streaked over the surface of the other half of the plate. The lid is sealed to the bottom half of the Petri dish by means of plasticine clay or similar material. The aerobe utilizes the free oxygen inside of the dish and eliminates carbon dioxide. The oxygen tension is soon reduced sufficiently to permit the growth of the anaerobe. The anaerobe is definitely benefited by the association while the aerobe is neither benefited nor injured.

Antibiosis or Antagonism.—There are species of bacteria that cannot live together, one either killing or inhibiting the growth of the other. It is probable that some metabolic waste product is produced which is without effect upon its producer but poisonous to its antagonist. This condition is termed antibiosis. Antibiosis may be defined as the living together of two organisms, one of which is distinctly injurious to the growth of the other species, resulting finally in its death.

Prescott and Baker (1904) observed that in a mixture of *E. coli* and *S. faecalis* the former organism increased at a more rapid rate than the latter during the first few hours, then the *S. faecalis* gradually gained the ascendency and finally outgrew the *E. coli* present. Savage and Wood (1917) worked with the same pair of organisms and made a similar observation. Hale (1926) noted that *Clostridium perfringens* outgrew *E. coli* in association and in many cases the latter organism was completely destroyed.

Greer and Nyhan (1928) prepared several pairs of organisms likely to be encountered in water supplies and observed the number of cells of each species at 24-hr. intervals. Their results showed that one member of a pair almost invariably outgrew or destroyed the other member. In most cases the final result depended upon the proportions of each organism present at the beginning of the experiment. If one organism is present in greater numbers than the second organism, the former will tend to gain the ascendency; if the second organism is present in greater numbers than the first organism, the reverse will be true.

Pseudomonas aeruginosa was found to be antagonistic to the growth of *Streptococcus faecalis*. When the two organisms were mixed *P. aeruginosa*

always outgrew *S. faecalis*. This occurred regardless of which organism predominated at the beginning. This is shown in Fig. 150.

Cultures or culture filtrates of some organisms are able to lyse or dissolve other bacterial species. *Bacillus subtilis*, *B. mycoides*, and several other organisms have been reported as being capable of secreting

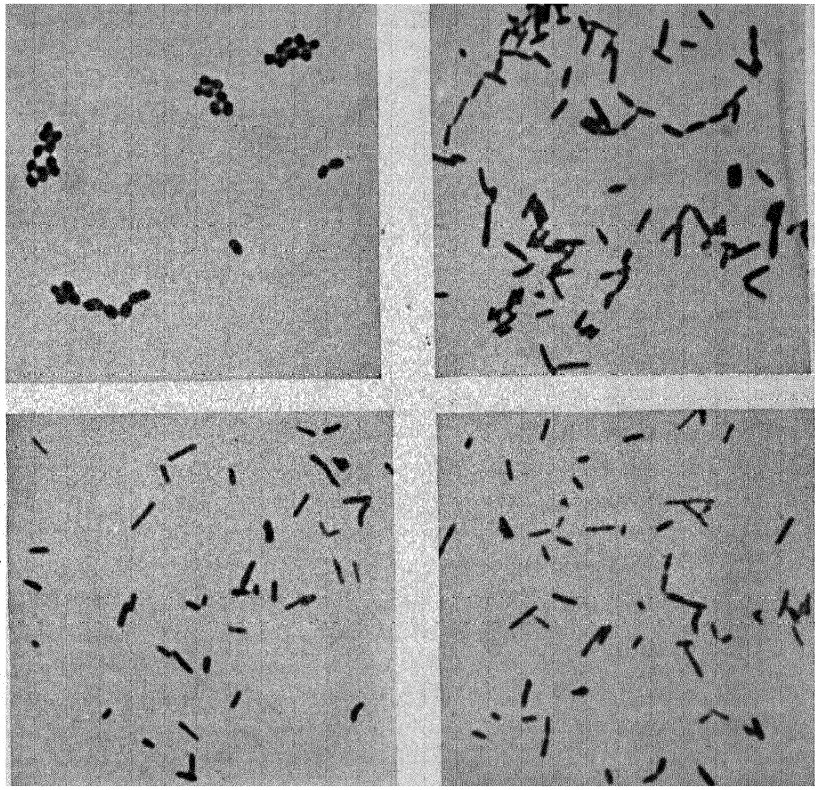

FIG. 150.—Antibiosis. *A*, *Streptococcus faecalis;* *B*, *Pseudomonas aeruginosa;* *C*, culture prepared by inoculating nutrient broth with one loopful of *S. faecalis* and 0.1 cc. of *P. aeruginosa* and incubating at 37°C. for 48 hr.; *D*, same as (*C*) except that 0.1 cc. of *S. faecalis* and one loopful of *P. aeruginosa* used. Regardless of which organism predominated at the start, *P. aeruginosa* outgrew the other.

a cytolytic substance having the power of dissolving cultures of *Diplococcus pneumoniae*, *Eberthella typhosa*, *Escherichia coli*, *Vibrio comma*, etc.

Dubos (1939) isolated a spore-bearing bacillus from the soil that was capable of dissolving living Gram-positive cocci. Autolyzed cultures of the organism were capable of dissolving living staphylococci, pneumococci, and various streptococci. He named the lytic factor gramicidin.

The addition of gramicidin to nutrient broth prevented the growth of Gram-positive cocci but was unable to retard the multiplication of any

of the Gram-negative bacilli. Streptococci that were incubated at 37°C. with gramicidin lost the ability to reduce methylene blue—indicating an inactivation of the dehydrogenase. This occurred before any morphological alteration of the cocci had taken place. Dubos believed that lysis was only a secondary process following some injury inflicted upon the cell.

Hoogerheide (1940), and McDonald (1940) isolated a substance from soil bacilli that inhibited encapsulation of Friedländer's bacillus (*Klebsiella pneumoniae*) and was also highly bactericidal for Gram-positive microorganisms. The elaboration of a lytic principle was by no means restricted to one organism but to a number of species. The most common spore formers, such as *B. subtilis*, *B. megatherium*, *B. mesentericus*, and *B. cereus*, excrete similar bactericidal products during growth in nutrient broth. A crystalline fraction obtained from the crude lytic material was found to be highly germicidal for many Gram-positive organisms when used in concentrations of from 0.00001 to 0.02 mg. per cubic centimeter of medium.

Waksman and Woodruff (1941) isolated an *Actinomyces* from soil that possessed strong bacteriostatic and bactericidal properties. The organism was found to belong to the chromogenic types of actinomycetes, which produced a dark brown to black pigment on peptone or protein media. They named the organism *Actinomyces antibioticus*.

An active substance was extracted from cultures of the organism by means of solvents. The substance was separated into two crystalline fractions which they designated actinomycin *A* and actinomycin *B*. The former fraction was found to be strongly bacteriostatic, whereas the latter was highly bactericidal. Actinomycin *A* was found to be bacteriostatic against all bacteria tested although Gram-positive species were more sensitive to the compound than Gram-negative forms. Later Waksman, Robinson, Metzger, and Woodruff (1941) reported that actinomycin was a powerful bacteriostatic agent against *Staphylococcus aureus, Clostridium perfringens* (*welchii*), *Streptococcus pyogenes*, and *Diplococcus pneumoniae* Type I. Over a 5-day period with or without the presence of 10 per cent serum, this bacteriostatic action slowly became bactericidal. The extract appeared to be more effective against pneumococci and streptococci than against staphylococci.

For further reading see the reviews by Waksman and Woodruff (1940), Waksman (1941), and the report by Stokes and Woodward (1942).

Synergism.—In recent years the phenomenon of synergism has received probably more attention than any of the other types of bacterial associations. The term was first suggested by Holman and Meekison (1926). Synergism may be defined as the joint action of two organisms

on a carbohydrate medium, resulting in the production of gas that is not formed by either organism when grown separately.

Sears and Putnam (1923) were probably the first to conduct systematic studies of synergism. They reported that many pairs of organisms were observed to produce gas from sugar media, which was not formed by either organism in pure culture. They explained the phenomenon by stating that one of the organisms of the pair was capable of forming acid while the other member produced the gas. The acid former degraded the carbohydrate and released a substance that was utilized by the

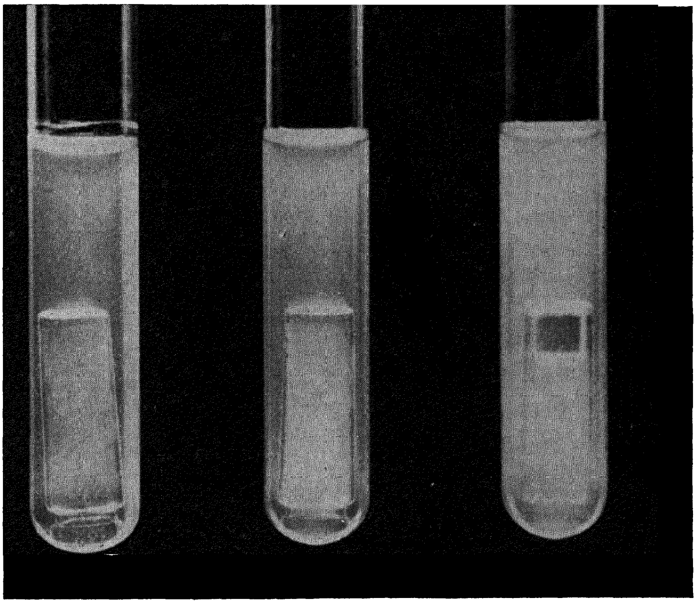

FIG. 151.—Bacterial synergism. Left to right, *Escherichia coli* grown in sucrose broth; *Staphylococcus aureus* in sucrose broth; a mixture of *E. coli* and *S. aureus* in sucrose broth with gas formation.

second organism, resulting in the production of gas. The substance attacked by the gas-forming member of the pair was not an end product of the action of the acid producer but an intermediate product of metabolism. Holman and Meekison (1926) confirmed the findings of Sears and Putnam and extended on their work. Their results for gas-forming pairs of organisms are given in Table 44. A typical example using sucrose broth and the organisms *Staphylococcus aureus* and *Escherichia coli* is shown in Fig. 151.

Castellani (1926) reported that *Eberthella typhosa* and *Proteus morganii* produced gas from some carbohydrates when grown in association but failed to do so when grown separately in pure cultures. Graham (1932) also worked with the same organisms and reported that the two species

TABLE 44.—BACTERIAL PAIRS THAT PRODUCE GAS IN ASSOCIATION

Carbohydrate	Organisms
Lactose	*Staphylococcus aureus* + *Salmonella schottmuelleri* *Streptococcus faecalis* + *Salmonella schottmuelleri* *Streptococcus faecalis* + *Salmonella choleraesuis* *Staphylococcus aureus* + *Proteus vulgaris* *Streptococcus faecalis* + *Proteus vulgaris* *Streptococcus faecalis* + *Salmonella paratyphi*
Sucrose	*Staphylococcus aureus* + *Escherichia coli* *Streptococcus faecalis* + *Escherichia coli* *Streptococcus equinus* + *Salmonella schottmuelleri* *Streptococcus equinus* + *Salmonella paratyphi* *Staphylococcus aureus* + *Salmonella paratyphi*
Mannitol	*Staphylococcus aureus* + *Proteus vulgaris* *Streptococcus faecalis* + *Proteus vulgaris* *Streptococcus pyogenes* + *Proteus vulgaris* *Shigella paradysenteriae* + *Proteus vulgaris* *Eberthella typhosa* + *Proteus vulgaris*

growing in association formed synergic gas from mannitol. *E. typhosa* produced a stable compound from mannitol, which was degraded by *P. morganii* to gas. *E. typhosa* grown alone produced an intermediate compound, which was found to be stable at 100°C. When a culture of *E. typhosa* was killed by heat and then inoculated with *P. morganii*, the intermediate compound was fermented with the liberation of gas. The gas was found to be the same as that produced from a mixture of the two organisms grown together.

Atkinson (1935) working with the same pair of organisms reported synergic gas from mannitol and from xylose. The amount of gas produced was found to be greatly increased by growth of the organisms in the presence of calcium carbonate. The function of the calcium carbonate appeared to be a neutralizing action on the acids produced by *E. typhosa* with the result that this organism remained in an actively growing condition for a longer period of time and continued to decompose the carbohydrate.

The phenomenon of synergism probably finds its greatest importance in the field of bacteriological water examinations. False, positive, presumptive tests in water analysis are frequently caused by the associated activities of two or more species of organisms. The opposite effect might also be obtained, namely, the failure of a pair of gas-producing organisms to form gas when grown in association. Greer and Nyhan (1928) reported the production of gas by pairs of nongas-forming organ-

isms. They concluded that synergic reactions in water examinations are not of common occurrence. The general tendency was for one member of a pair to inhibit or outgrow the other. This may be due to the elaboration of metabolic products by one organism detrimental to the other, to an increase in the hydrogen-ion concentration, to a higher growth rate by one of the members, etc. The presence or absence of gas does not necessarily mean that *E. coli* is present or absent in a water sample.

Atkinson and Wood (1938) stressed the importance of synergic pairs in producing false, positive, presumptive tests in water examinations. They isolated organisms growing in synergic association, one member being a nonlactose fermenter giving acid and gas from glucose and the other giving acid but no gas from lactose. Two pairs of organisms were isolated, both of which contained a nonlactose fermenter of the *Proteus* group. In one pair the organism was combined with *Streptococcus faecalis* and in the other pair with *Eberthella belfastiensis*. False, positive presumptives due to the combined action of *E. belfastiensis* and a *Proteus* type were encountered most frequently.

These are only a few examples of the various types of associations reported in the literature. Associations of bacteria are of frequent occurrence and greater emphasis should be placed on studies of this type in order that accurate interpretations may be made of the role played by organisms growing in their natural environment.

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CHAPTER XIX

BACTERIOLOGY OF AIR

GENERAL CONSIDERATIONS

Air is a mixture of gases composed of approximately 80 per cent nitrogen and 20 per cent oxygen. In addition it contains about 0.04 per cent carbon dioxide and traces of other gases such as neon, argon, and helium. All samples of air contain some water in the form of vapor or mist. Almost any sample contains suspended matter consisting of dust, bacteria, yeasts, molds, pollen grains, etc. Unlike the gaseous content, the suspended matter is not uniform but shows considerable variation.

The air in the vicinity of large industrial plants contains a considerable amount of dust, the nature of which depends upon the character of the operations in the plant. This may consist of minute metallic particles, carbon dust, fumes composed of gases and suspended matter, etc. All particles in suspension tend to settle out. This means that the air becomes purer as the altitude is increased.

Air is not a natural environment for the growth and reproduction of microorganisms. It does not contain the necessary amount of moisture and kinds of nutrients in the form utilizable by bacteria and other microscopic organisms. Air, therefore, possesses no bacterial flora. Yet organisms are found in air and their presence is of considerable importance economically and to public health.

Bacteria in Air.—Bacteria are introduced into the air by various forces. According to Chope and Smillie (1936) the principal source is from dust that contains dry vegetative cells and spores. These organisms are for the most part saprophytes, *i.e.*, forms that live on dead organic matter. They are of great importance to the canner, in the sugar refineries, in dairies, in the biological laboratories, etc. In short, they are the organisms responsible for contaminations from the air.

The species vary somewhat depending upon the locality. However, certain forms are quite uniformly present. Molds and yeasts are quite commonly found in the air and in some localities even outnumber the bacteria. These organisms produce spores that are capable of resisting unfavorable conditions for long periods of time. The aerobic spore-forming bacilli from the soil are found quite frequently in the air. The best-known member of this group is *Bacillus subtilis*. It is known as the hay bacillus and is probably the most common bacterial organism found

in nature. Its natural habitat is in the soil and on vegetation. It is a spore-forming organism, being capable of withstanding drying and other unfavorable environmental conditions for long periods of time. Sarcinae and micrococci are also found in air. The saprophytic, chromogenic, spherical organisms found in air usually belong to these two genera.

Number of Bacteria in Air.—The number of organisms present in the air is dependent upon the activity in the environment and the amount of dust stirred up. This means that there are more bacteria in the air during the day than during the night. The number of bacteria in the air of a room is increased by activity, as has been shown in schoolrooms. The numbers in dirty untidy rooms are greater than in clean rooms. Also, the air in small, poorly ventilated rooms shows a greater count than the air in larger rooms.

A rich, fertile, cultivated soil shows a higher viable count than a sandy, or clay, uncultivated soil. It follows that the air above the fertile, cultivated soil will contain more organisms than the air above the poor soil. Likewise the air above a bare surface contains more organisms than the air above land covered with vegetation. This means that where the earth is bare the organisms can be blown more easily into the air, owing to the fact that the earth is not protected from air currents.

The air in cities shows heavier bacterial pollution than air of the country. Also, bacteria are more numerous in the air of lowlands than in the air above mountains. The air in mid-ocean is practically free of organisms. It can be said that the air of uninhabited regions is comparatively free from living organisms.

Bacteria remain in air for varying periods of time, depending upon the speed of the air current, the size of particles on which they are attached, and the humidity of the air. Bacteria are slightly heavier than air and settle out slowly in a quiet atmosphere. A gentle air current is capable of keeping organisms in suspension almost indefinitely. This applies to organisms not attached to particles but existing in the free state. Bacteria attached to dust particles or in droplets of water settle out at a much faster rate.

A damp or humid atmosphere contains fewer organisms than a dry one owing to the fact that the organisms are carried down by the droplets of moisture. The air of a refrigerator is usually free from all organisms. Following snow or heavy rain, air is usually free of organisms. The water drops act mechanically by washing the atmosphere of all organisms. Therefore air during the dry summer months contains many more organisms than during the wet winter months. Gently expired air from the lungs is sterile. The moist passages of the upper respiratory tract remove the bacteria from the air. Cotton stoppers in pipettes are not necessary as far as contamination of the contents is concerned. The

stoppers are inserted as a protection against aspirating infectious or other material into the mouth.

Effect of Desiccation and Ultraviolet Light.—Desiccation and ultraviolet rays of the sun exert a pronounced germicidal action on bacteria and other organisms suspended in air. This applies equally as well to those organisms present in droplets of liquid, owing to the fact that the bacteria are soon freed from their surrounding moisture. Practically all suspended bacteria are killed by desiccation and light rays within a short period of time. The effect of drying and ultraviolet light on bacteria and their spores is discussed more fully on page 141.

ALTITUDES ATTAINED BY ORGANISMS

Proctor (1935) in his studies on the microbiology of the upper air found that organisms were able to attain considerable altitudes. To quote,

> The ability of these living microorganisms to attain altitudes of 20,000 feet or more through the chance action of air currents is particularly significant as it suggests the almost limitless possibilities of travel in a horizontal direction. The survival of such forms despite the many influences which are unfavorable to their existence is also significant in view of the length of time for which they may remain viable.
>
> The presence of pollen at high altitudes also indicates the importance of air as a vehicle for the transmission of wind-borne pollens over wide areas.
>
> The high dust counts obtained in comparison to the numbers of microorganisms is interesting in view of the various possible sources of dust, some of which, as from soil, might be also associated with high bacterial counts, while in other cases particles from smokestacks and industries might be sterile.

In a later communication Proctor and Parker (1938) described an improved apparatus and technique that they employed for upper-air investigations.

METHODS EMPLOYED FOR THE ENUMERATION OF BACTERIA IN AIR

A number of methods have been employed for the determination of the bacterial population of air. These are discussed in chronological order.

Pasteur (1861) appears to be the first to attempt a quantitative estimation of the bacteria in air by a very simple technique. He exposed a number of flasks of broth to various atmospheres for different periods of time and then sealed them. The flasks were incubated and those showing growth were counted.

Tyndall (1882) employed a table containing 100 apertures in the top. Into each aperture he placed a tube of nutrient broth. The table was then set in position and the cotton stoppers removed from the tubes, which were exposed to the air for a definite period of time. At the end of the

exposure period the cotton stoppers were replaced, the tubes incubated, and the contaminated tubes counted.

Miquel (1883) filtered air through glass wool. The glass wool was then washed with a known volume of sterile water and small aliquot parts added to many tubes of broth. The smaller the size of the inoculum showing growth in the broth, the larger the number of organisms present in the air. He attempted to add an inoculum small enough to contain only one organism. This meant that some tubes showed growth and others did not.

In 1881 Koch employed solid media poured into plates. He exposed the plates to the air for definite periods of time and then counted the colonies that developed on the agar after an incubation period.

Hesse (1884) attempted to make the method more quantitative by estimating the number of bacteria present per liter of air. He employed a glass tube 70 cm. long and 0.75 cm. in diameter, which had previously been coated with a layer of nutrient gelatin on the inside. A known volume of air was then drawn through the tube, after which it was incubated and the colonies counted. If all the organisms in the air sample were collected on the surface of the gelatin, the number of colonies that developed represented the count per liter of air.

Frankland (1887) found Hesse's method inaccurate, so he devised a procedure similar to that recommended by Miquel. A definite volume of air was first filtered through a plug of glass wool. The plug was next placed in sterile water and shaken until broken up. Measured amounts of the water were then mixed with melted agar and poured into plates. The plates were incubated and the colonies counted.

In 1888 Petri filtered air through a tube containing sand that had been previously screened through a 100-mesh sieve. The tube measured 9 cm. long and 1.6 cm. in diameter. A definite volume of air was drawn through the tube to collect the organisms on the sand. The sand was then shaken with sterile salt solution to suspend the bacteria, after which aliquot parts were pipetted into Petri dishes and mixed with melted agar. Sedgwick (1888) recommended sugar instead of sand. On being mixed with salt solution, the sugar dissolved, producing a suspension of bacteria without the presence of any insoluble sediment. In 1896 Ficker substituted ground glass for the sand.

Winslow (1908) modified the method of Hesse by drawing air into two 2½-liter flasks connected in series and containing a layer of nutrient agar in the bottom. A known volume of air was then drawn through the flasks, after which they were incubated and the colonies counted.

In 1909 the American Public Health Association officially adopted a modification of the sand method of Petri as the standard procedure for the bacterial analysis of air (Fig. 152). A layer of fine sand 1 cm. in

thickness is supported within a glass tube 70 mm. in length and 15 mm. in diameter upon a layer of bolting cloth, which in turn is supported by a rubber stopper. A tube 6 mm. in diameter and 40 mm. in length passes through a perforation in the rubber stopper. This latter tube is attached to an aspirator bottle. The upper end of the sand filter is closed with a cork stopper through which passes a tube of the same dimensions as the lower one. This tube is bent at an angle of 45 deg. to prevent dust and bacteria from falling into the sand filter. The sand is shaken into 10 cc. of sterile water and aliquot portions plated on nutrient agar.

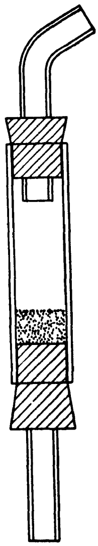


FIG. 152.—A modification of the Petri sand filter adopted by the American Public Health Association, 1909.

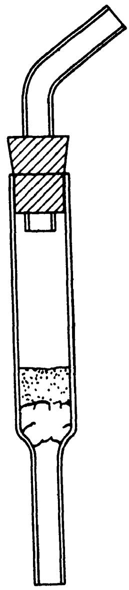


FIG. 153.—The Ruehle modification of the Petri sand filter adopted by the American Public Health Association, 1917.

Ruehle (1915*a,b*) recommended a modification of the Petri sand method which was adopted in 1917 by the Committee on Standard Methods for the Examination of Air (Fig. 153). The modification differs from the standard tube in no way except that the lower rubber stopper and the bolting cloth supports are eliminated and the same tube is fused into the larger one. The layer of sand is supported by a plug of cotton resting on the shoulder at the junction of the large and small tubes.

Browne (1917) suggested a modification of the official method in which the filter tube and washing tube are fused to each other at an angle of 90 deg. (Fig. 154). The whole operation can be performed in one piece of apparatus. During filtration of air the tube is held in the

position shown in *A*. After filtration the tube is turned, as shown in *B*. The sand passes from the filter tube into the diluting tube. The filter plug with the bolting cloth is replaced by a sterile stopper and then 10 cc. of sterile water pipetted into the diluting tube. The sand is thoroughly shaken and the water passed back and forth from the filter tube to the diluting tube. Aliquot portions are pipetted into Petri dishes and mixed with melted agar.

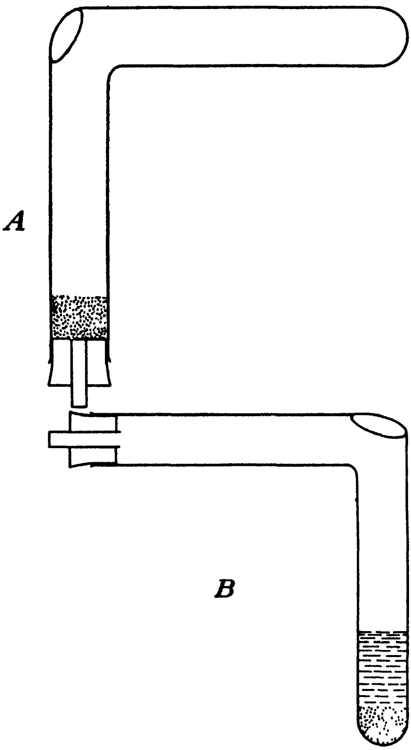


FIG. 154.—The Browne aeroscope for collecting bacteria.

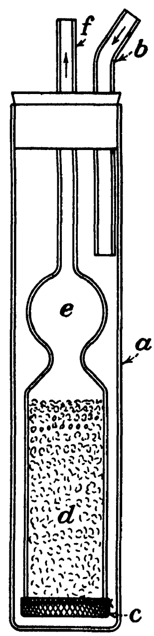


FIG. 155.—The McConnell modification of the Rettger aeroscope.

McConnell (1925) recommended the aeroscope shown in Fig. 155. The outer glass tube measures 240 mm. in length and 50 mm. in diameter, closed at the bottom. The top is fitted with a rubber stopper through which pass tubes *b* and *f*. The inner glass tube consists of a lower portion *d* measuring 105 mm. in length and 30 mm. in diameter. The upper portion is constricted to a diameter of 10 mm. and expands into a trap *e* measuring 35 mm. in diameter. This tube continues into the exit tube *f* measuring 100 mm. in length and 6 mm. in diameter. The lower end of the tube is covered with a piece of silk cloth *c* and tied in place by means of a piece of string. The intake tube *b* is 105 mm. in

length and 6 mm. in diameter, the upper portion of which is bent at an angle of 45 deg.

In preparing the aeroscope 25 cc. of sterile water is placed in container *a* and the openings *b* and *f* are plugged with cotton. The whole apparatus is then sterilized in the autoclave. Before using, the cotton stoppers are removed and tube *f* connected by means of rubber tubing to a suction pump. The air is drawn through tube *b* and through the water in tube *a* which in turn is raised by suction into tube *d*. The bacteria are suspended in the bubbling water. At the end of the test 1 cc. of the water is pipetted into a Petri dish and mixed with melted agar. The plate is incubated and colonies counted. The remainder of the water is measured in order to compute the dilution factor.

Wells (1933) described a simple instrument for enumerating bacteria in air, utilizing the principles of the centrifuge for their separation. The instrument operates in a manner similar to a milk clarifier, which separates heavier particles from a liquid. The rapid revolution of a glass cylinder about its vertical axis causes a current of air to enter through a central tube and to escape along a thin layer of nutrient agar deposited on the walls of the cylinder. Bacteria and other microorganisms suspended in the air are deposited on the agar. After incubation, visible colonies appear where individual bacteria were precipitated and can be counted.

The author stated that four independent operations are combined in one compact instrument. These are

1. Air flow is created.
2. The amount of air is measured.
3. The bacteria are collected.
4. The bacteria grow and can be counted on the collection medium without separate plating.

Hollaender and Dalla Valle (1939) described a funnel device for sampling air-borne bacteria (Fig. 156). The sampling device consists of a brass container with a removable bottom. The container is fitted with an inverted 60 deg., 3-in., glass funnel which sits approximately 1 cm. from the bottom of a standard-type Petri dish. The latter is placed in the lower portion of the container before use and is then screwed tightly against the washer indicated in the figure. The inside of the funnel and the rim are swabbed with alcohol before use. The air sample passes through the funnel stem, and the air-borne organisms and dust are impinged upon the medium placed in the Petri dish. The air sample is drawn by means of an ordinary impinger pump in series with a flowmeter. A sampling rate of 1 cu. ft. per minute was found to be the most effective.

Relative Merits of the Methods.—The plate method of Koch is probably the simplest procedure but is of no value from a quantitative standpoint. It does not indicate the number of organisms in a known volume of air. However, the method is commonly employed because of its simplicity and in many cases only relative results are desired. The results of plates exposed to different environments and incubated at 37°C. are shown in Fig. 157. It may be seen that the bacterial population increases as the activity of the atmosphere increases.

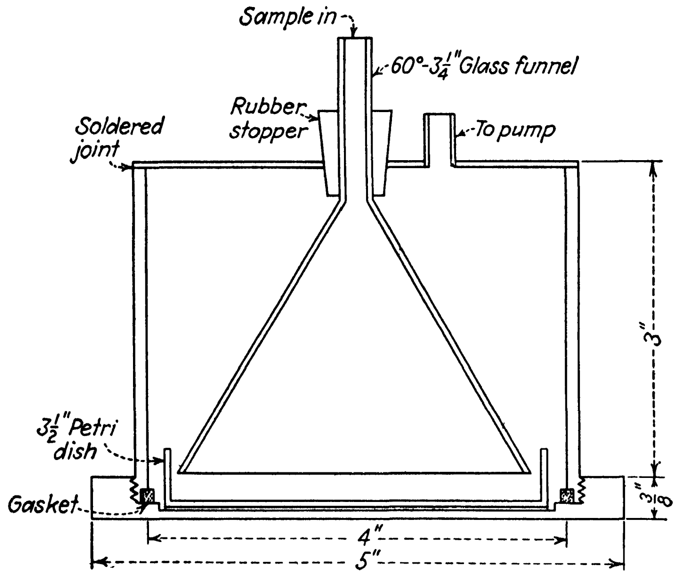
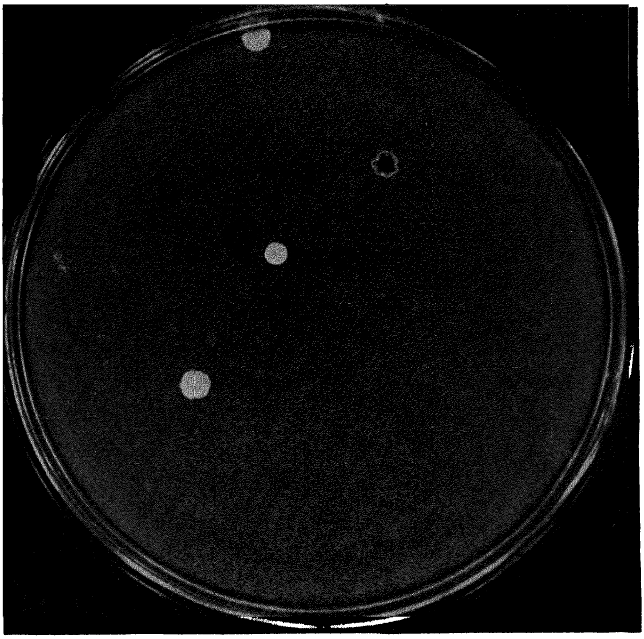


FIG. 156.—Funnel device used for sampling air-borne bacteria. (*After Hollaender and Dalla Valle.*)

The methods of Hesse and Winslow have the advantage that the organisms are collected directly on solid media without the necessity of an intermediate step. Frankland pointed out that the Hesse tube is not accurate. The apparatus of Winslow is too bulky to be portable.

The sand filters are simple in construction and portable but possess certain disadvantages: (1) The sand must be screened to a certain size and carefully sterilized to avoid caking. (2) The results do not give a true picture of the number of organisms in the air but only those which survived filtration and until the culture is prepared.

The Wells centrifuge is simple to operate, quickly manipulated, and portable. All testing is carried out in one step. The apparatus has a wide range of application, since any type of solid medium may be used, depending upon the types of organisms it is desired to cultivate.



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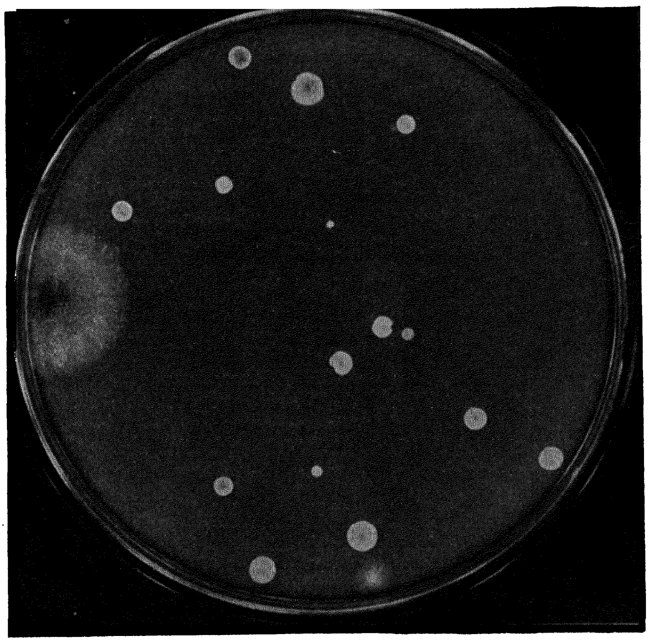

B

FIG. 157.—Air bacteria. *A*, colonies developing on an agar plate exposed to a quiet atmosphere (office). *B*, colonies developing on an agar plate exposed to an active atmosphere (laboratory).

Dalla Valle and Hollaender (1939) found their funnel device to be efficient and to compare favorably with the Wells centrifuge, giving slightly higher results when the bacterial population is low. The method is simple, portable, and all testing is carried out in one operation. Since it is one of the newest methods, sufficient comparisons with other procedures have not been reported.

IMPORTANCE OF STATE OF SUSPENSION IN SANITARY AIR ANALYSIS

Organisms in air are seldom in a free state but usually attached to floating particles such as dust, saliva, or carbon. The state of suspension plays a very important role on the settling velocity of bacteria in air. It is of great importance to distinguish between ubiquitous saprophytic soil organisms raised as dust and those from body tissues introduced into the air during the processes of coughing, sneezing, talking, and singing. The former probably do not have any pathogenic significance; the latter do.

The state of suspension of bacteria introduced into the air under these conditions is different. Organisms in the free state are slightly heavier than air and settle out very slowly in a quiet atmosphere. A gentle current is capable of keeping them in suspension almost indefinitely. Dust particles laden with bacteria settle out rapidly and remain in a quiet atmosphere for a relatively short period of time. Droplets expelled into the air during coughing and sneezing do not necessarily fall immediately to the ground within a short distance from their source. As droplets decrease in size, the surface exposed to air resistance becomes relatively greater when compared to the weight or gravitational attraction toward the earth. This means that the droplets fall slower and slower as the size becomes smaller and smaller. This is in accordance with Stokes' law which says that the velocity is proportional to the surface area of the droplet or to the square of its diameter. Stokes' law may be expressed by the following equation:

$$V = \frac{2}{9} r^2 \frac{(s - l)g}{u}$$

where V = velocity of settling.
r = radius of particle.
s = density of particle.
l = density of fluid.
g = acceleration of gravity.
u = viscosity of suspending fluid.

If the physical constants of air are known, the equivalent diameter can be computed from the settling velocity. The number of particles of uniform size and distribution that settle on unit area in unit time will

be equal to the product of the number per unit volume multiplied by falling velocity. The ratio of the number of particles settling on unit area divided by the number of particles per unit volume gives the settling velocity.

The rate of evaporation also depends upon surface area and becomes relatively more rapid as the size of the droplets becomes smaller. Some droplets are of such size that complete evaporation occurs in falling the height of a man. This droplet size has been estimated to be approximately 0.1 mm. in diameter. The residues of droplets of this size will float or drift with the slightest air currents and become, in effect, a part of the atmosphere itself.

Air infections may then be said to occur by means of two types of droplets, depending upon their size. Droplet infection proper applies to droplets larger than 0.1 mm. in diameter, which rapidly settle out a short distance from their source before drying occurs. The other type of droplet may be called air-borne infection and applies to the dried residues of infected droplets (droplet nuclei) derived from droplets less than 0.1 mm. in diameter. The time they remain suspended in air depends upon the activity of the atmosphere. Droplet infection may become air-borne infection when large droplets evaporate in settling to the ground and then are lifted into the air as dust. It can be seen that droplet infection remains localized and concentrated and that air-borne infection may be carried long distances and is dilute.

Jennison and Edgerton (1940), by means of high-speed photography, showed that droplets discharged in coughing and sneezing may move at a rate as fast as 100 ft. per second. Such a velocity in dry air would result in nearly instantaneous evaporation, producing droplet nuclei. They also showed that the great majority of sneeze droplets, before appreciable evaporation occurred, measured 0.1 to 2 mm. in diameter.

For a more complete discussion see Wells (1934), Wells and Wells (1936), Phelps (1940), and Wells, Phelps, Robertson, and Winslow (1940).

Air-borne Infections.—Newer techniques developed during the past ten years have caused an increased interest in the subject of air bacteriology. It was formerly supposed that air played an insignificant role in transferring infection from one person to another. This view is no longer tenable since it has been definitely established that air is capable of transmitting infections and that infections transferred in this manner may be of frequent occurrence. This is especially true in closed spaces such as rooms, offices, theaters, and halls.

Wells (1935, 1938) in his researches on air-borne infections came to the following conclusions:

1. During coughing and sneezing, minute droplets containing microorganisms from infected surfaces may be ejected into the air.

2. Most of these droplets are sufficiently small to evaporate before they can settle to the ground, leaving suspended in the air minute residues.

3. These nuclei, in which the microorganisms remain viable for considerable periods, may drift in air currents like particles of cigarette smoke.

4. The air breathed commonly by the various persons congregated in a room or other enclosed space can thereby transfer organisms from one person to another and plant them upon the susceptible tissues of the respiratory tract.

Wells and Brown (1936) reported that eight ferrets contracted influenza from virus that had been recovered from experimental suspensions of influenza virus in air. The virus was recovered from air after being suspended for at least 30 min.

Pressman (1937) exposed evaporating dishes containing sterile saline solution for a period of one week in four tuberculosis sanatoria. The dust residue was collected, centrifugated, digested, and stained or cultured. The results were overwhelmingly positive for the presence of the tubercle bacillus.

Buchbinder, Solowey, and Solotorovsky (1938) found that *alpha* hemolytic streptococci were widely distributed in the air of enclosed places of congregation and of the open spaces of New York City. A large majority of these organisms appeared to be of nasopharyngeal origin. The organisms were present most frequently and in greatest numbers in the air of school buildings. An apparent relationship was noted between the type of room and degree of occupancy and the numbers found. The largest counts were obtained from occupied assembly and classrooms.

In another communication the same workers (1938) reported that the occurrence of *beta* hemolytic streptococci in air was relatively infrequent. They succeeded in isolating 52 strains, all but 5 from indoor locations.

These are only a few of the many results reported in the literature. For an excellent discussion of the subject of air-borne infections read the reports by Rentschler and Nagy (1942), Solowey, Solotorovsky and Buchbinder (1942), Wells, Wells, and Mudd (1939), Wells and Henle (1941), and Jennison and Turner (1941).

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CHAPTER XX

BACTERIOLOGY OF WATER

Water receives its bacterial flora from air, soil, sewage, organic wastes, dead plants and animals, etc. This means that almost any organism may be found in water. Most of the bacteria soon die, but a few are able to adapt themselves to the new environment. The latter organisms constitute the natural flora of water.

The great majority of the bacteria found in nature live on dead organic matter. They are harmless saprophytes and belong to the so-called metatrophic group of organisms. Saprophytes are particularly rich in that diffuse layer of decomposing plant and animal matter known as humus. It is, therefore, the metatrophic organisms that are found for the most part in water.

Natural waters are commonly grouped into four well-marked classes: (1) the atmospheric waters, (2) the surface waters, (3) the stored waters, and (4) the ground waters.

Atmospheric Waters.—Rain and snow are included under the atmospheric waters. Sometimes these may contain considerable numbers of bacteria. After a snow or heavy rain the atmosphere is washed nearly free of organisms so that many sterile plates, each inoculated with 1 cc. of water, may be obtained.

Surface Waters.—As soon as the raindrops and snowflakes touch the earth, they become quickly contaminated by the microorganisms in the soil. These are then known as surface waters. The extent of the contamination is dependent upon the numbers of organisms in the soil and, also, upon the kinds and quantities of food materials dissolved out of the soil by the water. The bacterial counts of surface waters are apt to show great variations. This is particularly true in the fall and spring, the seasons of heavy rains and melting snows. The washoff from the soil may upset the existing equilibrium in the surface water, resulting in considerable variation in the flora and bacterial count.

The first result of a mild rain is greatly to increase the bacterial contamination of a body of water. A prolonged rain exerts an opposite effect, owing to the fact that after the main impurities have been removed from the upper layers of the soil the subsequent rainfall acts merely as a diluent of the body of water. Rivers usually show their highest count during the rainy period.

Stored Waters.—The effect of storage is notably to decrease the numbers of organisms in water. The forces that tend to produce bacterial self-purification now come into play. These are sedimentation, activities of other organisms, ultraviolet light, temperature, food supply, and perhaps osmotic effects.

Bacteria have a specific gravity slightly greater than that of distilled water, which means that they will slowly settle in a still body of water. However, the greatest factor responsible for the sedimentation of bacteria is their attachment to suspended particles. The suspended particles in settling mechanically remove the organisms from the upper layers of the water.

Protozoa present in waters play an important role in decreasing the numbers of bacteria. These microscopic animals easily engulf large quantities of dead or living bacteria. The protozoa remain alive, provided the water is well aerated. In the absence of sufficient dissolved oxygen the protozoa gradually disappear.

Direct sunlight is very toxic to vegetative bacterial cells and even to spores if the action is sufficiently prolonged. Diffuse light is less effective as a sterilizing agent. In a water supply the toxicity of ultraviolet rays is inversely proportional to its turbidity. This means that the light rays are practically without action in a turbid water. In a clear water, however, ultraviolet rays may be effective for a depth of from 1 to 2 meters.

Increasing temperatures exert a harmful effect upon the survival of some organisms in water, especially those producing disease. On the other hand, multiplication of certain soil and intestinal forms may actually occur when the temperature of the water is increased. Rector and Daube (1917), Savage and Wood (1917), Winslow and Cohen (1918), and Winslow and Falk (1923) all reported slight increases in the numbers of *E. coli* in various kinds of stored waters. More recently Bigger (1937) showed that *E. coli* was capable of multiplying in water from various sources that had been autoclaved previous to inoculation and then incubated at 37°C. In some instances the numbers of organisms present were more than 10,000 times the original count. Cultures in autoclaved water kept at 22°C. showed higher counts and a greatly prolonged period of positive cultures as compared with those kept at 37°C. Raw waters also showed an increase in bacterial numbers when stored at 22°C. and 37°C. but not so great as when autoclaved water was used.

Increasing the food supply usually results in increasing the numbers of bacteria. On the other hand, certain toxic substances such as acids and bases produce marked reductions in the numbers of viable organisms. Various dissolved gases such as carbon dioxide and hydrogen also show a toxic effect. Environmental factors generally produce marked fluctua-

tions in the bacterial counts. Apparently this is not due to any one factor but to a group of factors acting as a whole.

Obviously, all the factors that operate to decrease the numbers of bacteria in water will be more effective with an increase in time. This may be repeated mathematically as follows:

$$\log \frac{N_1}{N_2} = kt$$

where N_1 = number of organisms at the beginning.
N_2 = number of organisms at the end.
t = time.
k = a constant that varies with temperature and other environmental factors.

Ground Waters.—Ground waters are, in general, relatively free from bacteria, because of the filtering action of the earth through which the waters have penetrated. This filtering action removes not only most of the bacteria but also any suspended organic food particles. Deep wells contain usually fewer organisms than water from shallow wells, owing to the deeper layers of filtering material.

QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF WATER

The usual methods employed in the laboratory for the quantitative, bacteriological examination of water give only a fraction of the total count. Many of the organisms are classed with the nitrifying and strict anaerobic bacteria, which either do not grow on the usual laboratory media or multiply very slowly. Certain obligate, parasitic bacteria fail to grow in the absence of rich animal fluids. The autotrophic organisms that live on inorganic salts are unable to grow in the presence of so much organic matter present in the usual media. The result is that most of the bacteria found in water escape detection. From 20 to 70 times as many organisms have been enumerated by the direct or microscopic count as compared to the agar-plate method. Larger bacterial counts may be obtained by employing certain special, culture media.

No great error is introduced by failure to obtain the total bacterial count of water. The sanitary bacteriologist is interested not in such organisms as the anaerobic spore formers, nitrifying and autotrophic bacteria, and pathogenic organisms, but in a group of rapidly growing, rich food-loving organisms found in sewage. Most of these organisms belong to the *Escherichia* and *Aerobacter* divisions of the colon group.

Method.—The method for the quantitative macroscopic bacterial count consists in placing a measured amount of the water sample in a Petri dish and mixing with sterile, melted agar. After the agar has solidified, the plate is incubated at a temperature of 37°C. for 24 hr.

The colonies are counted and the count is expressed as the number of colonies per cubic centimeter of water that develop on the plate.

The sample of water is collected in a clean and sterile 100-cc. glass-stoppered or screw-cap bottle. The neck and top of the bottle are covered with a parchment paper cap and tied in place with a piece of string. The bottle is then sterilized in a hot-air sterilizer at a temperature of 170°C. for at least 1 hr. The purpose of the covering is to keep the neck and stopper of the bottle free from contaminating microorganisms.

In order to obtain a representative sample from a tap, the water should be allowed to run for at least 5 min. to remove any contaminating organisms present around the opening of the faucet. Also, changes in bacterial content are liable to occur in small pipes; some species tend to die and others to multiply. The bottle is grasped with the right hand. The stopper is removed with the left hand, holding it by the paper covering. After the sample is collected, the stopper with covering is carefully replaced, to avoid contamination, and the paper tied in place with string. The fingers must not touch the inside of the neck or stopper, otherwise contamination of the contents may occur and lead to an erroneous result.

In sampling a still body of water, the cap is first removed with the left hand. The bottle is plunged mouth downward to a depth of about 1 ft., then inverted. When filled, it is removed and stoppered. If any current exists, the mouth of the bottle should be directed against it in order to avoid the introduction of bacteria from the fingers.

After a sample of water has been collected and stored, a rapid change in the bacterial content takes place. The numbers of organisms usually show marked increases. In some cases the increase in numbers is gradual; in others it is very rapid. The increase in numbers is due to a multiplication of the typical water bacilli. Disease and other organisms, whose natural habitat is the intestinal tract of man and animals, tend to die very rapidly.

An increase in bacterial numbers is greatly accelerated by an increase in temperature. Because of the rapid bacterial changes that may take place in bottled samples, even when stored at temperatures as low as 10°C., all samples should be examined as quickly as possible. "Standard Methods for the Examination of Water and Sewage" (1936), published by the American Public Health Association, recommends that the time allowed for storage or transportation of water samples and the beginning of the analysis should not be more than 6 hr. for impure waters and not more than 12 hr. for relatively pure waters. The samples during the period of storage should be kept at a temperature between 6 and 10°C.

The number of bacteria present in a sample should not exceed 300 per cubic centimeter. If the number is greater than this, dilutions should be prepared. Also, if more than 300 organisms per cubic centimeter

are present, many of them will fail to grow, owing to the inhibitory action of the waste products secreted by those organisms developing first.

Various factors influencing the numbers of colonies developing on agar plates include composition of the medium, reaction of the medium, temperature of incubation, period of incubation, and presence of an abundant supply of oxygen and moisture. Unless such factors are controlled, variable counts will be obtained.

WATER-BORNE DISEASE

The most important bacterial diseases transmitted by means of water are dysentery, cholera, and typhoid. In this country typhoid is the most important one. Since they are intestinal diseases, the causative agents are found in the intestinal contents. Therefore the presence of sewage in a water supply means that one or more of these disease organisms may be present and that the water is potentially dangerous for human consumption.

Theoretically it would be better to examine a water supply for the presence of disease organisms to determine its potability from a bacteriological standpoint. However several difficulties enter into such a procedure. Chief among these may be mentioned (1) the length of time disease organisms remain viable in water and (2) the numbers encountered in a water supply.

Disease organisms die very rapidly in water. It is doubtful if organisms causing the above diseases are able to survive beyond one or two weeks. Most of the organisms probably die in a few days. They may remain alive longer in water containing considerable organic matter and in water that is cool. Epidemics caused by the presence of the intestinal disease organisms in water usually appear within a few days of each other, indicating that the infections occurred at about the same time.

The numbers of disease organisms encountered in a water supply are usually exceedingly small. If only one person in a community is suffering from typhoid fever and the discharges from that individual are mixed with the sewage from all individuals, the high dilution would make it practically impossible to isolate the disease organisms from a convenient sample of water. If, on the other hand, many persons are suffering from typhoid fever, the concentration of bacilli in sewage might be high enough to make isolations relatively easy but special culture media would be required for this purpose. For these reasons attempts to isolate disease organisms directly from water supplies are rarely practiced.

Presence of Escherichia coli in Sewage.—*E. coli* was first isolated by Escherich (1885) from the feces of an infant. It was shown later

to be a normal inhabitant of the intestinal tracts of man and animals. Since it is present in the discharges from all individuals, with rare exceptions (Parr 1938), tests for the potability of a water supply are based on the presence or absence of this [illegible] Its presence in water does not mean that disease organisms are present but that they might be present. In other words, all sewage-polluted waters are potentially dangerous.

The procedures employed for the bacteriological examination of water do not give necessarily positive tests for *E. coli* alone but for other closely related organisms that possess little or no sanitary significance. The most important of these is *Aerobacter aerogenes.* This organism is occasionally present in the intestines of man and animals but it is chiefly a soil inhabitant. For this reason the term "colon group" will be used rather than *E. coli.* The colon group includes all aerobic and facultative anaerobic, Gram-negative, nonspore-forming, rod-shaped organisms that ferment lactose with the production of acid and gas. The separation of these two groups is discussed on page 427.

THE PRESUMPTIVE TEST

The first step in water examinations is known as the presumptive test. The test consists in placing graduated amounts of water in a series of lactose-fermentation tubes each containing at least twice as much medium as water. It is the usual practice to employ five fermentation tubes each containing 10 cc. of water, one tube containing 1 cc. of water and another with 0.1 cc. of water. The tubes are incubated at 37°C. for 48 hr.

The formation of 10 per cent or more of gas in 24 hr. at 37°C. constitutes a positive, presumptive test. It is presumptive evidence for the presence of members of the colon group. The presence of less than 10 per cent of gas in 48 hr. constitutes a doubtful, presumptive test and requires further examination. The absence of gas formation after an incubation period of 48 hr. constitutes a negative, presumptive test and no further tests need be performed. The water may be considered satisfactory from the bacteriological standpoint.

False Positive Presumptive Tests.—A positive, presumptive test does not necessarily mean that members of the colon group are present. In most cases it is true, but there are exceptions. False, positive, presumptive tests are caused by (1) the presence of other organisms capable of fermenting lactose with the production of acid and gas and (2) bacterial associations or synergism.

In an important series of papers Greer (1928*a*,*b*,*c*,*d*), Greer, Tonney, and Nyhan (1928), Greer, Noble, Nyhan, and O'Neil (1928), and Greer and Noble (1928) discussed completely the lactose-fermenting organisms that do not belong to the colon group. They showed that the organisms

most frequently encountered in the Chicago water supply included *Clostridium perfringens* (*welchii*), *Bacillus aerosporus*, *Streptococcus faecalis*, members of the Friedländer group (Klebsiella), *Pseudomonas aeruginosa*, Houston's leather bacillus, and several species of *Erwinia*. With the exception of Houston's leather bacillus and several species of *Erwinia*, all the organisms have been isolated from feces. Atkinson and Wood (1938) came to a similar conclusion. They found that false, presumptive tests were caused by *S. faecalis*, *C. perfringens*, organisms of the genus *Proteus*, and *B. coli anaerogenes*.

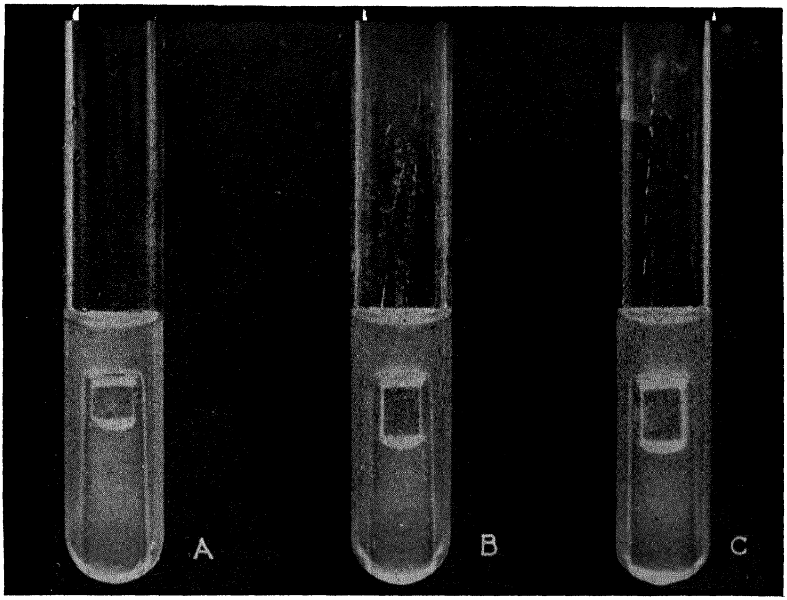


FIG. 158.—Lactose-broth fermentation tubes. *A*, 1-cc. water sample; *B*, 0.5-cc. water sample; *C*, 0.1-cc. water sample. All tubes show fermentation of the lactose with the production of acid and gas.

Positive, presumptive tests are frequently caused by a type of bacterial association known as synergism. Bacterial synergism may be defined as the joint action of two organisms on a carbohydrate resulting in the production of gas that is not formed by either organism when grown separately. This is discussed in greater detail on page 396.

Elimination of False, Presumptive Tests.—Probably the most important procedure employed for the elimination of false, positive, presumptive tests is to incorporate a very small amount of a suitable triphenylmethane dye in the lactose-broth medium. In most cases synergism is caused by a Gram-positive and a Gram-negative organism growing together. A concentration of dye just sufficient to prevent the growth of Gram-positive organisms will have no effect on the growth of the Gram-

negative bacteria. This will result in the elimination of a synergistic reaction. False, positive, presumptive tests caused by the presence of gas-forming, Gram-positive aerobes and anaerobes will also be eliminated by this procedure.

THE CONFIRMED TEST

The lactose-broth fermentation tubes showing a positive or doubtful presumptive test are utilized in the following procedures. It is customary to employ the tube showing at least 10 per cent of gas from the smallest

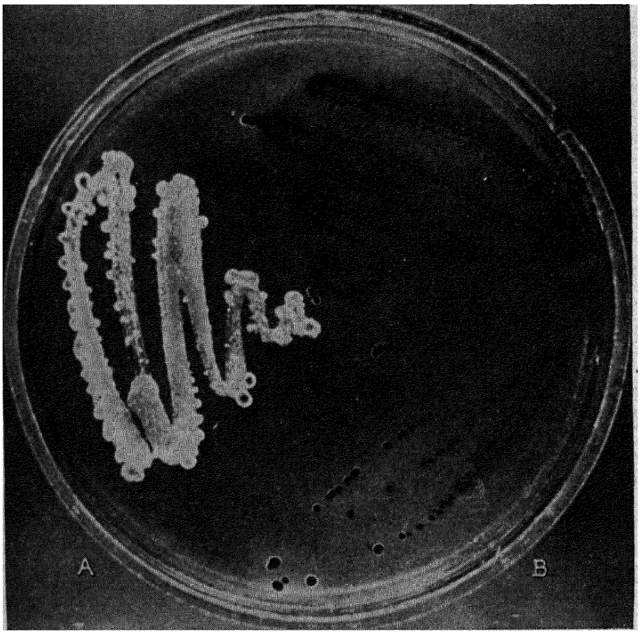


FIG. 159.—Eosin methylene blue agar plate. *A*, *Aerobacter aerogenes* colonies do not produce a metallic sheen; *B*, *Escherichia coli* colonies produce a metallic sheen.

amount of water tested. For example, if all the tubes show the presence of acid and at least 10 per cent of gas, the test is confirmed only from the tube containing 0.1 cc. of water.

A loopful of the culture is streaked over the surface of an Endo or an eosin methylene blue (E.M.B.) agar plate. The plate is incubated at 37°C. and examined at the end of 24 or 48 hr., depending upon the reaction obtained.

If typical colonies appear on the plate, the test is considered positive. If no typical colonies appear in 24 hr., the plate should be reincubated for another 24 hr. The absence of typical colónies at the end of 48 hr. does not mean that the test is negative for members of the colon group since some strains form atypical colonies. Regardless of whether typical or

atypical colonies appear, it is necessary to continue with the test as described under Completed Test, page 427.

The presence of typical colonies on Endo or E.M.B. agar plates after 24 hr. constitutes a positive, confirmed test (Figs. 159 and 160). If no typical colonies appear at the end of 24 or 48 hr., the plates are retained for the completed test.

Eosin Methylene Blue Agar Medium.—Eosin methylene blue agar plates are prepared by adding the two dyes eosin and methylene blue to melted lactose agar and pouring 10-cc. amounts into Petri dishes.

When typical strains of *E. coli* are streaked over the surface of this medium and the plates incubated at 37°C. for 24 hr., the colonies show dark centers and possess a characteristic greenish metallic sheen. Typical colonies of *A. aerogenes*, on the other hand, show brown centers, and a metallic sheen is rarely observed.

Their colonial characteristics, according to Levine (1918, 1921) are given in Table 45.

TABLE 45

	E. coli (1)	*A. aerogenes* (2)
Size	Well-isolated colonies are 2 to 3 mm. in diameter	Well-isolated colonies are larger than *E. coli;* usually 4 to 6 mm. or more in diameter
Confluence	Neighboring colonies show little tendency to run together	Neighboring colonies run together quickly
Elevation	Colonies slightly raised; surface flat or slightly concave, rarely convex	Colonies considerably raised and markedly convex; occasionally the center drops precipitately
Appearance by transmitted light.	Dark almost black centers, which extend more than three-fourths across the diameter of the colony; internal structure of central dark portion difficult to discern	Centers deep brown; not as dark as *E. coli*, and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies
Appearance by reflected light.	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen	Much lighter than *E. coli*, metallic sheen not observed except occasionally in depressed center when such is present

1. Two other types of *E. coli* colonies have been occasionally encountered. One resembles the type described except that there is no metallic sheen, the colonies being wine colored. The other type of colony is somewhat larger (4 mm.), grows effusely, and has a marked crenated or irregular edge, the central portion showing very distinct metallic sheen. These two varieties constitute about 2 or 3 per cent of the colonies observed.

2. A small type of *A. aerogenes* colony, about the size of the *E. coli* colony, which shows no tendency to coalesce has been occasionally encountered.

The medium is relatively stable; prepared plates have been kept in the refrigerator for a week or even longer before use and found to be satisfactory. The plates should not be exposed unnecessarily to the light, otherwise toxic substances may be formed in the medium (see page 144).

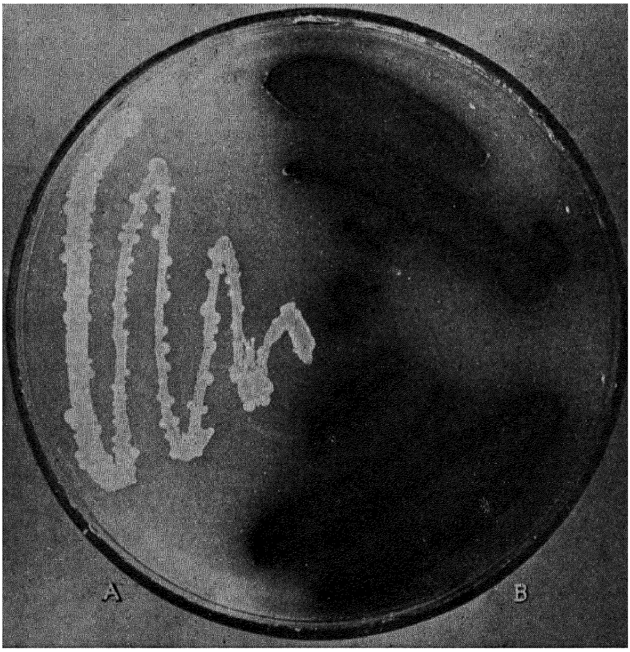


FIG. 160.—**Endo agar plate.** *A*, growth of *Aerobacter aerogenes* does not produce a metallic sheen; *B*, growth of *Escherichia coli* produces a metallic sheen and darkens the medium.

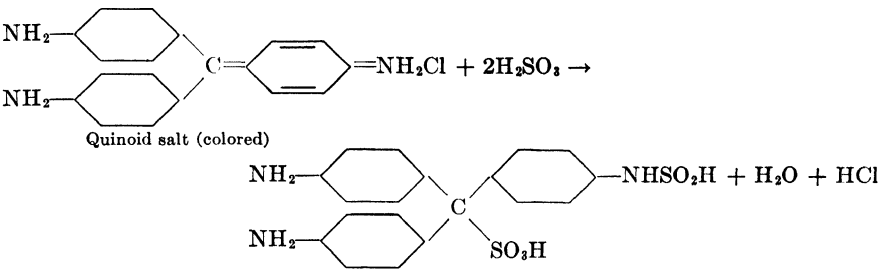
Endo Agar Medium.—The use of this medium was first advocated by Endo (1904). Endo agar plates are prepared by adding basic fuchsin, previously decolorized with sodium sulfite, to melted lactose agar and 10-cc. amounts poured into Petri dishes.

When typical *E. coli* is streaked over the surface of this medium, the red color of the dye is restored and metallic, gold-like colonies appear. If the fuchsin-sulfite solution is added after 24, 48, and 72 hr. of incubation, no reaction takes place. The substance responsible for the reaction is detected only when the bacteria are grown in the presence of the sulfite. It is believed that Endo agar acts as a trapping agent for acetaldehyde, which is the compound responsible for the characteristic

reaction. Acetaldehyde is an intermediate compound in the fermentation of lactose.

Basic fuchsin consists largely of a mixture of *p*-rosaniline and rosaniline hydrochlorides. On the addition of a sulfite the compounds are decolorized. According to Margolena and Hansen (1933) the dyes react with sulfurous acid as follows:

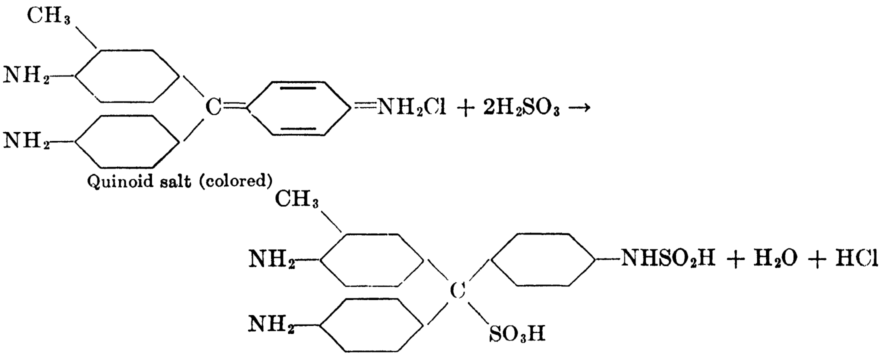
Pararosaniline hydrochloride:



Quinoid salt (colored)

Nonquinoid compound (colorless)

Rosaniline hydrochloride:



Quinoid salt (colored)

Nonquinoid compound (colorless)

When an aldehyde such as formaldehyde is added to the decolorized compound, it reacts with the sulfite or sulfurous acid to form an addition compound. The dye is then released from the combination, resulting in the restoration of the red color. Margolena and Hansen showed that the restored dye was different chemically from the original compound, being more purplish in color. The process appears to be more complicated than just a removal of the sulfite and a liberation of the basic fuchsin dye.

The results obtained by Neuberg and Nord (1919) in their studies on the fermentation of glucose by *E. coli* may be offered as additional evidence in support of the aldehyde hypothesis. They found that acetaldehyde accumulated without question when sodium sulfite was added and that none was found when the sulfite was omitted.

Their reactions are as follows:

$$\underset{\text{Glucose}}{C_6H_{12}O_6} \rightarrow \underset{\text{Methylglyoxal hydrate}}{2CH_3{\cdot}CO{\cdot}C\begin{smallmatrix}\diagup H\\ -OH\\ \diagdown OH\end{smallmatrix}}$$

$$CH_3{\cdot}CO{\cdot}C\begin{smallmatrix}\diagup H\\ -OH\\ \diagdown OH\end{smallmatrix} \begin{cases} \nearrow \underset{\text{Lactic acid}}{CH_3{\cdot}CHOH{\cdot}COOH} \\ \searrow \underset{\text{Acetaldehyde}}{CH_3{\cdot}CHO} + \underset{\text{Formic acid}}{HCOOH} \end{cases}$$

$$HCOOH \rightarrow H_2 + CO_2$$

$$2CH_3{\cdot}CHO + H_2O \rightarrow \underset{\text{Acetic acid}}{CH_3{\cdot}COOH} + \underset{\text{Alcohol}}{CH_3{\cdot}CH_2OH}$$

In summary it may be stated that the restoration of the red color is due to the production of acetaldehyde, which is capable of forming an addition product with sulfites. The metallic gold-like sheen imparted to the colonies is due to the precipitation of the liberated dye by the organic acids (such as lactic acid). The restored dye is not the same as the original fuchsin but possesses a more purplish color.

Liquid Confirmatory Media.—One or more of several liquid media may be employed for the confirmed test in place of the Endo or E.M.B. agar plates. These are brilliant green, lactose bile broth; crystal violet broth; fuchsin, lactose broth; and formate, ricinoleate broth. The liquid media are all equivalent for the confirmed test, but since waters vary considerably in their microflora, one medium may give results superior to the others. Therefore a selection of any one of them should be based upon the correlation of the confirmed tests thus obtained with a series of completed tests.

All the media are liquid, contain lactose, and are dispensed in test tubes with inverted vials. Their composition is such that they are supposed either to eliminate entirely or reduce to a minimum the growth of organisms not members of the colon group.

The test is made by transferring a loopful of culture from the lactose-broth fermentation tube showing a positive, or doubtful, presumptive test to a tube of one of the liquid confirmatory media. The formation and presence of gas in any amount within 48 hr. at 37°C. constitutes a positive, confirmed test.

The Committee on Standard Methods conducted an exhaustive study in an attempt to determine the comparative, practical utility of these four liquid confirmatory media when employed in routine water analysis. The results of the tests were reported by McCrady (1937). Twenty-one laboratories situated in various parts of the United States and Canada collaborated in the study. The results obtained indicated that brilliant green, lactose bile broth was the most generally satisfactory medium of

those tested. Also, the results compared very favorably with those obtained from the standard methods completed test.

COMPLETED TEST

The purpose of the completed test is to determine (1) if the colonies appearing on an Endo or an E.M.B. agar plate are again capable of fermenting lactose with the production of acid and gas and (2) if the organisms transferred to an agar slant show the morphological and tinctorial picture of members of the colon group.

At least one typical colony or, if no typical colonies are present, at least two atypical colonies considered likely to be members of the colon group are each transferred to a lactose fermentation tube and to an agar slant. The fermentation tube is incubated at 37°C. and examined at the end of 24 and 48 hr. The agar slant is incubated at 37°C. for 24 hr. and then examined microscopically by the Gram technique. The formation of gas in any amount in the fermentation tube within 48 hr. and the presence of Gram-negative, nonspore-forming rods on the agar slant shall be considered a positive, completed test for members of the colon group. The absence of gas or failure to show the presence of rods answering to the above description in a gas-forming culture shall constitute a negative completed test.

The presumptive, confirmed, and completed tests make no distinction between so-called fecal and nonfecal types. The American Public Health Association feels that any attempt to evaluate a drinking water on the basis of a distinction between the above two types is unwarranted. However, the procedures that follow make an attempt to distinguish between fecal *E. coli* and nonfecal *A. aerogenes*. The tests are employed in many laboratories but are not to be regarded as official in any sense of the word.

DIFFERENTIATION OF FECAL FROM NONFECAL MEMBERS OF THE ESCHERICHIA-AEROBACTER GROUP

Results of investigations in this country have demonstrated that the colon group may be divided into two subgroups, typical members of which differ in many respects but especially in their nitrogen and carbohydrate metabolism. These two subgroups are known as the *E. coli* and *A. aerogenes* divisions of the colon group.

When members of the *Escherichia-Aerobacter* group are inoculated into lactose-broth fermentation tubes, it may be observed that some cultures produce more gas than others. A differentiation of the organisms reveals the fact that typical strains of *E. coli* rarely produce more than 25 per cent of gas in the inverted vial and that typical strains of *A. aerogenes* produce from 75 to 100 per cent of gas. This indicates

that there are distinct differences in the carbohydrate metabolism of the two subgroups.

Rogers, Clark, and Davis (1914), Rogers, Clark, and Evans (1914, 1915), and Rogers, Clark, and Lubs (1918) demonstrated that typical strains of *E. coli* produced carbon dioxide and hydrogen in approximately equal amounts and that typical members of the *A. aerogenes* division produced about twice as much carbon dioxide as hydrogen. The former are known as the low-ratio organisms ($CO_2/H_2 = 1$) and the latter as the high-ratio fermenters ($CO_2/H_2 = 2$).

The high-ratio organisms (*A. aerogenes*) are only occasionally found in the intestinal contents of man and animals (about 6 per cent). They are normally present in the soil and on grains. For this reason very little sanitary significance is attached to their presence in a water supply. On the other hand, the low-ratio organisms (*E. coli*) are rarely found on grains and in the soil but constitute one of the predominating organisms found in the intestinal contents of man and animals. They are only occasionally found in localities not showing recent fecal pollutions.

It is generally stated that members of the *A. aerogenes* division of the colon group are considerably more viable in a water supply than are members of the *E. coli* division. It is true that there is some difference but this is not so great as was formerly supposed. Parr (1937) inoculated typical *E. coli* into sterile tap water containing bits of string and found that the organisms were still viable after 2 years, 4 months, and 4 days. Several other experiments gave essentially the same type of results. He concluded that

> *Escherichia coli* and *Aerobacter aerogenes* remain viable in a variety of environments for long periods of time. The difference in viability of coli and aerogenes when studied outside the body is not as great as has been supposed. When both organisms coexist in an environment, certainly in feces, aerogenes survives longest.

Voges-Proskauer Test.—This reaction was first observed by Voges and Proskauer (1898) in connection with their studies on the organisms of the hemorrhagic septicemia group. They found that the addition of potassium hydroxide to a culture of the organisms under observation resulted in the development of a pink color, if the tube was allowed to stand at room temperature for 24 hr. or longer.

The chemistry of the reaction was worked out by Harden and Walpole (1906), Harden (1906), and Harden and Norris (1912*a*,*b*). They found that distinct differences existed in the carbohydrate metabolism of typical *E. coli* and *A. aerogenes*. The fermentation of glucose by the two organisms yielded the products shown in Table 46.

TABLE 46

Product	Per cent by weight of glucose fermented	
	A. aerogenes	*E. coli*
Alcohol	17.10	12.85
Acetic acid	5.10	18.84
Succinic acid	2.40	5.20
Formic acid	1.00	0.00
Lactic acid	5.50	31.90
Carbon dioxide	38.00	18 10
Total	69 10	86.89
Ratio, CO_2/H_2	2 40	0.83

The figures show that 87 per cent of the carbon is accounted for in the case of *E. coli*, but only 69 per cent in cultures of *A. aerogenes*. Harden and Walpole found that the discrepancy was due to the formation by *A. aerogenes* of 2:3 butylene glycol ($CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3$) and acetylmethylcarbinol ($CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3$) but not by *E. coli*. The acetylmethylcarbinol in the presence of potassium hydroxide and air is further oxidized to diacetyl ($CH_3{\cdot}CO{\cdot}OC{\cdot}CH_3$) which, in the presence of peptone, gives an eosin-like color. The constituent of peptone responsible for the eosin-like color is the guanidine nucleus [$NH{:}C(NH_2)$-$NH{\cdot}R$] in the amino acid arginine.

The Voges-Proskauer test appears to possess considerable sanitary significance because it distinguishes to a high degree between typical fecal (low-ratio) and typical nonfecal (high-ratio) members of the colon group.

Methyl Red Test.—Michaelis and Marcora (1912) noted that cultures of *E. coli* fermented lactose with the production of acids until a pH of about 5.0 was reached. This acidity was sufficient to prevent further growth of the organisms. They concluded that the final hydrogen-ion concentration of cultures of *E. coli* was a physiological constant. This same principle applies to any fermentative organism.

Clark (1915) and Clark and Lubs (1915) confirmed the work of Michaelis and Marcora. They stated that the metabolism of members of the colon group can be so controlled that the hydrogen-ion concentration of cultures of one subgroup can be made to diverge widely from those of the other subgroup. From a given amount of sugar *E. coli* will produce more acid than *A. aerogenes*. This is due to the fact that *E. coli* does not produce 2:3 butylene glycol and acetylmethyl carbinol and that *A. aerogenes* does. The amount of fermentable carbohydrate that is just sufficient for *E. coli* to produce its maximum acidity is inade-

quate for *A. aerogenes* to produce its limiting hydrogen-ion concentration. The *E. coli* organisms will be stopped in their growth, whereas *A. aerogenes* will exhaust the sugar and, being insufficient for them to produce their limiting hydrogen-ion concentration, will then attack the nitrogenous constituents of the medium for both structure and energy. The reaction of the medium becomes progressively more alkaline.

The amount of buffer present greatly influences the final hydrogen-ion concentration attained by an organism when grown in the presence of a fermentable substance. The greater the buffer content the smaller will be the final hydrogen-ion concentration (higher pH). This means that as fermentation is prolonged, metabolic products other than acids accumulate to produce an increased toxic effect on the organisms.

The medium used for the test contains 0.5 per cent glucose sufficiently buffered with dibasic potassium phosphate and peptone to give a limiting hydrogen-ion concentration of about pH5.0 when inoculated with typical *E. coli*. The final hydrogen-ion concentration of cultures of typical *A. aerogenes* will be at a much higher pH. The methyl red indicator used in the test is turned red by cultures of *E. coli* and orange or yellow by *A. aerogenes*.

Uric Acid Test.—The Voges-Proskauer and methyl red tests are based on a difference in the carbohydrate metabolism of the members of the colon group. The tests do not consider changes that take place by action of the organisms on the nitrogenous constituents of the medium.

Koser (1918) observed that cultures of typical *A. aerogenes* were capable of utilizing uric acid when added to a synthetic medium as the only source of nitrogen. Cultures of typical *E. coli* were unable to attack the compound. The result was that *A. aerogenes* multiplied and grew luxuriantly and *E. coli* failed to develop. The results correlated almost 100 per cent with the methyl red and Voges-Proskauer tests.

Later Chen and Rettger (1920) found that xanthine could be substituted for uric acid with the same result. They obtained almost 100 per cent correlation with the methyl red and Voges-Proskauer tests. Xanthine yields uric acid on oxidation. The formulas are given on page 252.

Sodium Citrate Test.—Koser (1923, 1924), in his studies on the utilization of the salts of organic acids by members of the colon group, found that the organisms could be separated into two subgroups on the basis of their action on sodium or potassium citrate.

Typical fecal *E. coli* was unable to utilize sodium or potassium citrate when added to a synthetic medium as the only source of carbon. On the other hand, *A. aerogenes*, or the strictly soil types, utilized sodium citrate readily. A synthetic medium containing citrate as the only source of carbon is employed in the test.

Koser showed that the test for citrate utilization correlated more closely with the source of the organisms than did any of the other differential tests. Parr (1938) isolated a coliform organism that failed to show growth at first on citrate agar but produced a few colonies after 3 or 4 days. In a check of over 5000 strains isolated from fresh and stored fecal specimens this same result occurred only 29 times, indicating that the phenomenon is not of common occurrence.

RELATIONSHIPS OF COLIFORM ORGANISMS

Studies of recent years have emphasized the complexity of the colon group. The general practice followed in classifying members of the colon group is to designate as *Escherichia* those strains which are indole and methyl red positive, do not produce acetylmethylcarbinol, and are incapable of utilizing sodium citrate as the only source of carbon. Strains classified as *Aerobacter* are indole and methyl red negative, produce acetylmethylcarbinol, and are capable of utilizing sodium citrate as the only source of carbon. Parr (1936) coined the mnemonic "imvic" to designate these four reactions. According to this system the symbol for typical *E. coli* is "+ + − −" and for typical *A. aerogenes* "− − + +." Using these four characters as a system of classification, Parr (1938*a*) showed that 16 different organisms are possible. These are given in Table 47.

TABLE 47.—IMVIC CHARACTERS OF COLIFORM ORGANISMS FROM FRESH FECES

Indole	Methyl red	Voges-Proskauer	Sodium citrate	Type
+	+	−	−	Typical *E. coli*
−	+	−	−	Atypical *E. coli*
+	−	−	−	Atypical *E. coli*
+	+	+	−	Intermediate
+	+	−	+	Intermediate
−	+	−	+	Intermediate
+	−	+	−	Intermediate
+	−	−	+	Intermediate
−	+	+	−	Intermediate
+	+	+	+	Intermediate
+	−	+	+	Intermediate
−	+	+	+	Intermediate
−	−	−	+	Atypical *A. aerogenes*
−	−	+	−	Atypical *A. aerogenes*
−	−	+	+	Typical *A. aerogenes*
−	−	−	−	

The most commonly occurring types are − + − −, + + − −, − + − +, and − − + +.

According to Parr (1938*a*) coliform intermediates may be defined as

> those organisms of the group which have one or more *coli* and one or more *aerogenes* characteristics and some, principally the typical fecal form, produce hydrogen sulfide. Essential *E. coli* characteristics are the positive indol and methyl red reactions. Those most characteristic for *A. aerogenes* are the positive Voges-Proskauer and citrate utilization reactions.

Changes in the "imvic" characteristics produce changes in the colonial appearance of the organisms. Many types of colonies have been recognized, ranging from flat, round, smooth, glistening colonies typical of *E. coli*, to raised, markedly convex, confluent, mucoid colonies typical of *A. aerogenes*.

Parr concluded that

> there is great variability in the so-called normal coliform flora and in the flora of the same individual from day to day. Stool specimens may contain no coliform organisms at all and citrate utilizing forms may be present to the exclusion of the colon bacillus. No hope is offered for short cuts or simplifications in the bacteriology of water and foods through the domination of a typical intestinal form. The complexity of the coliform group is emphasized. It seems impossible to translate the work of the past into terms used today, making necessary the duplication of much work. Doubt is expressed as to the primary etiology of atypical, coliform organisms and it is suggested that the ecological point of view should receive more emphasis in intestinal bacteriology.

Stuart, Griffin, and Baker (1938) also recognized the complexity of the colon group and suggested that it be divided into three subgroups: *Aerobacter*, intermediate, and *Escherichia* on the basis of their indole and Voges-Proskauer reactions.

For excellent reviews see the monographs by Parr (1937, 1938*b*), Stuart, Wheeler, and Griffin (1938), Parr (1939), Griffin and Stuart (1940), and Stuart, Baker, Zimmerman, Brown, and Stone (1940).

SLOW LACTOSE-FERMENTING ORGANISMS OF THE COLON GROUP

Parr (1938*c*) stated that the flora of fresh, fecal specimens may consist of coliform bacteria (*Escherichia*, intermediates, *Aerobacter*), enterococci (*Streptococcus*), obligate, spore-forming anaerobes (*Clostridium*), nonspore-forming anaerobes (*Bacteroides*), *Staphylococcus*, *Lactobacillus*, thermophilic organisms (*Streptococcus*), *Bacillus*, aerobic spore formers (*Bacillus*), *Pseudomonas*, *Proteus*, *Alcaligenes*, members of the Friedländer group (*Klebsiella*), molds, yeasts, algae, and spirochaetes.

Parr showed that the flora of an individual differs from time to time even though the person is in normal health and the diet relatively stable. Occasionally coliform bacteria could not be detected but at other times coliform organisms (*Aerobacter*, intermediates), but not *E.*

coli, were present. It can be seen that fresh fecal specimens may be encountered that can pollute water without detection by the bacteriological procedures now in use. Fortunately such exceptions are rare and offer very little difficulty in sanitary interpretations of bacteriological results.

The bacterial flora of long-stored feces is different from that of fresh feces. Jordan (1926) found that shortly after feces left the body increase in bacterial numbers took place, followed later by a decrease. This occurred at all temperatures but was most rapid at 37°C. He found that increase in numbers was not due to *E. coli* but to other organisms. *E. coli* was eventually eliminated in stored feces. Parr reported that about 14 per cent of all fresh fecal specimens contained only *E. coli*. He concluded that when the original fresh specimens contained only *E. coli* no amount of storage brought about the presence of any other type of coliform organism. When the original fecal specimen contained both citrate-utilizing, coliform organisms and *E. coli*, the citrate utilizers (*A. aerogenes*, intermediates, etc.) multiplied at a faster rate than the *E. coli*, eventually replacing them. At the same time there was an increase in strains that fermented lactose slowly or with acid production only.

Several investigators have reported the transformation of late lactose-fermenting organisms of the colon group into rapidly fermenting strains by frequent subculture in lactose broth or nutrient broth. The "imvic" characteristics showed that the organisms belonged to either the *Escherichia* or *Aerobacter* subgroups. Late lactose fermenters have been isolated from water supplies and from feces of both healthy individuals and those ill with diarrhea.

Hershey and Bronfenbrenner (1936) found that a rapid lactose fermenter could be changed to a nonlactose-fermenting form by inoculation into a sodium succinate medium. They suggested that late lactose-fermenting organisms may not be identical with other lactose-fermenting members of the colon group. Stokes, Weaver, and Scherago (1938) reported that they were able to convert late lactose fermenters into rapid fermenting forms and back again to late fermenters. They concluded that the strains studied were members of the *Escherichia* and *Aerobacter* divisions of the colon group.

Occasionally members of the colon group become pathogenic for humans. Ziegler (1939) brought forth evidence to show that pathogenicity may be related to the late lactose-fermenting types. He succeeded in isolating a late lactose fermenter in pure culture from the blood stream of an adult male. Others have reported similar findings.

Dulaney and Smith (1939) examined 400 stool specimens and found that 20 per cent contained slow lactose fermenters. They showed that

in most cases slow lactose fermenters required from 2 to 5 days to produce acid and gas in fermentation tubes. Daily transfers to lactose broth accelerated the rate at which the sugar was fermented. They also isolated slow lactose fermenters from urine, from blood cultures, and from cases of diarrhea.

McCrady (1939) submitted a questionnaire to about 30 laboratory workers and found that the majority of them favored inclusion of the slow lactose fermenters in the coliform estimate in judging the sanitary quality of a water supply. He concluded,

> Slow lactose fermenters are found, although in small proportion, in fresh feces, in greater proportion in stored feces, and occasionally in discharges from cases of gastro-intestinal disturbance; the presence of these organisms even in natural waters, therefore, cannot always be dismissed as of negligible sanitary significance. Furthermore, since other organisms contained in the sample may reduce the amount of gas produced in lactose broth by typical coliform organisms, the volume of gas produced cannot be accepted as a sure indication of the type of organism present.
>
> It is evident, therefore, that the great majority of the laboratory workers are opposed to excluding slow lactose fermenters from the coliform group of organisms. In view of the various facts and arguments that may be adduced to support their position, it is suggested that, until sufficient evidence to the contrary is presented, water laboratories are well advised to include in their routine coliform estimates, all coliform organisms confirmed from primary lactose broth.

Stuart, Mickle, and Borman (1940) suggested a classification for the slow, lactose-fermenting organisms, placing all strains under the general name of "aberrant coliforms." Their conclusions concerning the importance of slow fermenters were essentially the same as those of McCrady.

Summarizing, it may be stated that at one time the general tendency was to regard late lactose fermenters as attenuated forms of colon organisms, indicating an old pollution, having no sanitary significance. At the present time available evidence seems to point to the fact that the slow fermenters are forms that have departed from the normal types but should be regarded as possessing considerable sanitary significance.

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CHAPTER XXI

BACTERIOLOGY OF MILK AND MILK PRODUCTS

MILK

Milk is a secretion of the mammary glands of mammals and is considered the most satisfactory single food preparation elaborated by nature.

According to Van Slyke and Bosworth (1915), the constituents of milk may be placed in three groups on the basis of their solubilities. Some of the constituents are present in true solution, some partly in solution and partly in suspension or colloidal solution, and others are present entirely in colloidal solution. This is discussed on page 321.

Color of Milk.—The color of milk is due to the presence of the yellow pigment carotene. Carotene exists in at least three isomeric forms. These are α-carotene, β-carotene, and γ-carotene. Another pigment closely related to carotene is cryptoxanthin, which occurs in yellow corn. All these pigments are precursors of vitamin A. One molecule of β-carotene is capable of yielding two molecules of vitamin A; one molecule of each of the others give only one molecule of vitamin A.

Carotene is found in hay, grass, green leaves, some fruits, carrots, etc. The carotene content of cows' milk is dependent upon the carotene content of the ration. Not all the carotene of the ration is converted into vitamin A. When cows consume carotene-containing foods, some of the pigment is converted into vitamin A and some is found unchanged in the milk. Vitamin A is colorless whereas carotene is yellow.

NORMAL SOURING OF MILK

Reaction of Fresh Milk.—Milk when freshly drawn may show considerable variation in reaction. As a general rule, the pH is slightly acid, ranging from about 6.3 to 7.2 with an average at about 6.75. The pH fluctuates at different stages of the milking operation. The fore milk is usually the lowest in acidity, the middle milk the highest, and the strippings are between the two.

Changes in the Reaction of Milk.—Unsterilized milk on standing rapidly ferments with the production chiefly of lactic acid from the lactose of the medium. The first stage is believed to be a hydrolysis of the lactose to one [illegible] glucose and one of galactose. In the second stage the hexoses are fermented to lactic acid.

It is generally stated that an acidity in milk is first detected by taste at a pH of about 6.0. As the acid concentration continues to increase, it eventually causes a precipitation of the casein. This is said to occur when the pH reaches 4.78 to 4.64. Boiling produces a curdling of milk at a much higher pH (lower acidity). The acidity continues to rise until the concentration is sufficient to prevent the growth of the organisms. The lactic acid produced in the milk prevents the growth of most types likely to be present and thus acts as a preservative.

Molds and yeasts are capable of growing in soured milk, utilizing some of the acid, and causing a decrease in the acidity. Conditions now

FIG. 161.—*Streptococcus lactis*, the cause of normal souring of milk.

become favorable for the rapid decomposition of the milk proteins by the growth of putrefactive bacteria. As a rule, several weeks are required for putrefaction to occur. The utilization of the acid occurs at a faster rate if the milk is placed in shallow well-aerated layers. This is the general cycle of changes that occur in raw milk when it is allowed to stand at ordinary temperature.

Streptococcus lactis.—The organism responsible for the normal souring of milk is known as *Streptococcus lactis*. Several varieties of the organism have been isolated, which show differences in the flavor produced, character of the fermented milk, rate of acid formation, rate of litmus reduction, and in other ways. Hammer and Baker (1926), Stark and Sherman (1935), and others suggested a number of varieties of the organism: (1) *S. lactis* var. *maltigenes*, (2) *S. lactis* var. *hollandicus*,

(3) *S. lactis* var. *anoxyphilus*, and (4) *S. lactis* var. *tardus*. Other varieties include (5) *S. amylolactis*, (6) *S. raffinolactis*, and (7) *S. saccharolactis* (Orla-Jensen and Hansen, 1932). Further proof of the similarities of the various strains and varieties of *S. lactis* was furnished by Sherman, Smiley, and Niven, Jr. (1940). They produced species-specific grouping sera and found that such sera gave good precipitin reactions with the extracts of all strains of *S. lactis* tested.

S. lactis does not occur in the udders of cows. This was shown by Rogers and Dahlberg in 1914. More recently Stark and Sherman (1935) succeeded in isolating *S. lactis* repeatedly from certain plants. They suggested that plants may represent the natural habitat of the organism and that it would seem likely that surviving strains would sometimes be found in the feces of animals. The organism can be obtained from the coat of the cow. Since it is normally present in cow dung, it is believed that this is the agent responsible for the contamination of milk.

S. lactis is Gram-positive and may appear in pairs, in short chains, or in long chains (Fig. 161). The organism is spherical but sometimes appears slightly elongated in the direction of a chain. The organism produces dextrolactic acid. No gas is formed. Occasional strains have been noted that fail to ferment lactose. In broth cultures the final pH falls between 4.0 and 4.5. The organism will not grow at pH9.5 but will grow at 9.2. It grows at 10°C. or lower and at 40°C. but not at 45°C. The optimum temperature is about 30°C. Litmus is completely reduced (decolorized) before the milk is curdled.

QUANTITATIVE EXAMINATION OF MILK

It is doubtful if normal udders of cows are ever sterile. Organisms are present in abundance in freshly drawn milk. The first milk drawn contains the largest number of organisms, the middle milk contains a smaller number, and the strippings contain the smallest number. The comparatively high count of the fore milk is due to the washing out of the easily removable organisms present in the milk passages. The numbers washed out become less and less during the milking process. It is the general practice to discard the first portions of the milk containing the highest bacterial counts. It has been shown that the rejection of the fore milk decreases the bacterial counts on an average of about 4 per cent. However, most of the organisms found in milk are chiefly those which gain entrance during the operations of milking and handling. It would seem, therefore, that the contamination of milk by bacteria is largely preventable.

Two methods are followed for making a quantitative bacteriological examination of milk: (1) the agar plate method, and (2) the direct microscopic method.

Agar Plate Method.—This method consists in preparing a series of dilutions of the milk, pipetting 1 cc. amounts into a series of Petri dishes, mixing with agar previously melted and cooled to 45°C., and incubating the plates at 37°C. for 48 hr. The factors that influence plate counts include temperature of incubation, period of incubation, medium used for plating, etc. Unless a standard procedure is followed the results obtained cannot be compared with those from other laboratories. Results reported by Abele (1939) and others indicate that an incubation temperature of 32°C. gives higher plate counts than one of 37°C.

Objections to the Plate Method.—Objections to the method are numerous. Pathogenic organisms are not detected. If the organisms grow, they cannot be distinguished from the nonpathogenic species by appearance. Also many pathogenic species, such as *Mycobacterium tuberculosis*, fail to grow under such conditions.

The number of colonies appearing on agar plates do not represent all the organisms in milk. Many of the organisms fail to develop on the agar medium. Anaerobic organisms do not find conditions favorable for growth. This means that no single medium is capable of giving growth of all viable organisms likely to be found in milk. Also a temperature of 37°C. is not favorable for the growth of all organisms. Shaking the sample does not break up all the clumps or groups of bacteria. Chains of streptococci usually remain intact and record as only one colony. The colony counts represent only a fraction of the total bacterial content of milk. Because of the long incubation period the milk is usually consumed before information on the number of bacteria present is obtained. Therefore, agar plate counts should be regarded as estimates rather than as exact numbers.

Direct Microscopic Method.—This method consists in spreading 0.01 cc. of milk over an area of 1 sq. cm. on a glass slide, allowing the film to dry, removing fat, staining film, and examining under the microscope.

The method possesses several advantages over the plate method. Results can be obtained quickly, usually in about 15 or 20 min. Since less work is required, more samples can be examined by this method than by the plate procedure. The amount of equipment necessary is much less than by the plate method. The slides can be preserved as a permanent record and examined whenever occasion arises, whereas the plates must be examined and discarded. Some idea of the morphological types present can be obtained from slide preparations. This is frequently of great value in determining the cause of the bacterial count. Microscopic examination reveals the presence of leucocytes and other body cells in milk. An excessive number of leucocytes indicates a

diseased condition of the udder. The slide method gives a better quantitative determination than the agar plate method.

Objections to the Microscopic Method.—An important disadvantage to the slide method is that it cannot be used on pasteurized milk. Dead cells are not easily distinguished from living cells. However, it does give important information as to the number of organisms present before pasteurization.

Another disadvantage is that unless the milk sample contains a high count the microscopic method may be the source of considerable error. A large factor is used for converting the number of bacteria per field to the number per cubic centimeter of milk. Significant errors in the average number of organisms per field are not likely to be of great importance when bacteria are numerous in milk. However, in low-count milk a considerable error may be introduced. This is especially true where many fields may be seen which show no bacteria and some fields which may show a cluster or chain of organisms so that the variation per field is great.

Comparison of Counts by the Two Methods.—The microscopic method gives much higher counts than the agar plate method. The differences between the counts by the microscopic and plate methods are considerably greater on samples showing low bacterial counts than on those showing high counts. The organisms in low-count milk represent external contaminants that fail to develop on agar, whereas those organisms in high-count milk are forms that have developed in the milk. Also, low-count milk usually shows a greater percentage of the organisms in clumps than high-count milk. It is generally stated that the ratio of the microscopic count to the plate count is, on the average, about 4 to 1. Therefore, this ratio is generally used to compare the results of one method in terms of the other, although this is not necessarily true.

Grading of Milk.—The number of organisms permissible in various grades of milk varies considerably, depending upon standards set up by local public health authorities.

The highest grade of milk is known as certified milk. Such milk is safeguarded at every step in its production, collection, and distribution. It is produced according to rules and regulations formulated by medical milk commissions established in a number of localities in the United States. The rules and regulations deal with such matters as the cleanliness of the barnyard and dairy buildings, quality of the water supply, sterilization of all utensils used in handling the milk, the periodic examination of the cows for the presence of tuberculosis and other diseases, and the examination of the milkers and others concerned with the handling of the milk. Persons suffering from contagious diseases or carriers of such organisms are not permitted to be employed in certified milk dairies.

Milk collected under conditions not so carefully controlled is frequently graded as *A*, *B*, or *C*. The ratings are based upon the bacterial count of milk and also upon the hygienic conditions under which it is produced. The standards of the various grades reported here are those set up by the U.S. Public Health Service Milk Ordinance (1935). These are as follows:

Raw Milk:

1. *Certified.*—This must conform to standards set up by American Association of Medical Milk Commissions. This varies for different localities but the usual standard is that the count must not go above 10,000 organisms per cubic centimeter. All milk having a count in excess of this number must be placed in one of the following grades:
2. *Grade A.*—The count must not exceed 50,000 bacteria per cubic centimeter.
3. *Grade B.*—The count must not exceed 200,000 bacteria per cubic centimeter before delivery.
4. *Grade C.*—The count must not exceed 1,000,000 bacteria per cubic centimeter before delivery.
5. *Grade D.*—Milk that does not meet the requirements of grade *C* must be labeled, "cooking only."

Pasteurized Milk:

1. *Grade A.*—Grade *A* pasteurized milk is grade *A* or *B* raw milk that has been subjected to one of the pasteurization processes, cooled, and bottled. The average count must not exceed 30,000 bacteria per cubic centimeter before delivery.
2. *Grade B.*—Grade *B* pasteurized milk is grade *C* raw milk that has been subjected to one of the pasteurization processes, cooled, and bottled. The average count must not exceed 50,000 bacteria per cubic centimeter before delivery.
3. *Grade C.*—This is pasteurized milk that does not meet the requirements of grade *B* pasteurized milk and must be labeled "cooking only."

Samples for bacteriological examinations are collected by inspectors or other officials. At least 10 cc. of well-agitated milk or cream is collected and placed in a sterile sample bottle. The bottle should be of such size that only about two-thirds of it is filled. This provides sufficient air space for vigorous agitation in order to obtain a uniform suspension of organisms before plating the milk. The sample is quickly cooled to between 0 and 4.4°C. and held at this temperature until analyzed. The average bacterial plate count means the logarithmic average of the plate counts of the last four consecutive samples taken upon separate days.

INFLUENCE OF TEMPERATURE UPON THE KEEPING QUALITY OF MILK

The number of organisms in milk at the outset depends upon the degree of care exercised in its production and collection. After collection the numbers increase rapidly unless milk is stored at low temperatures. The temperature at which it is stored determines to a large extent the bacterial count and the microflora of milk.

Germicidal Property of Milk.—Freshly drawn milk contains substances that are capable of exerting a bactericidal action. These substances are destroyed at high temperatures but the temperature required varies for different organisms. For example, the germicidal property of milk for *Aerobacter aerogenes* was almost destroyed at 60°C. for 20 min., whereas the same temperature and time did not destroy the bactericidal substances for *Eberthella typhosa*. However, a temperature of 70°C. for 30 min. markedly decreased the toxicity of milk for *E. typhosa*. Many examples of a similar nature may be cited from the literature.

For many organisms, at least, low-temperature pasteurization produces very little, if any, destructive action on the bactericidal property of milk. In the flash or high-temperature method this property may be largely destroyed. Therefore, bacteria increase at a more rapid rate in strongly heated milk than in raw milk or milk heated at low temperatures.

Milk Held in Frozen Condition.—If milk is held below the freezing point (−0.55°C.), no multiplication of organisms occurs. Since the milk is frozen throughout, there is no chance for the organisms to obtain their nutrients. Milk treated in this manner shows a decrease in bacterial numbers. If the milk is slowly frozen, there is a gradual precipitation of casein and an immediate destruction of the fat emulsion. When such milk is brought back to room temperature, especially if it has been frozen for some time, it does not regain its normal consistency.

Milk Held Just above Freezing.—If raw milk, or milk pasteurized at temperatures below 70°C., is kept at 0 to 5°C. for 24 hr., the plate count decreases. After a lapse of about one week there is an increase over the original plate count of the milk. At the same time the number of organisms capable of liquefying gelatin increases. This continues until enormous numbers are present. Some of the organisms are acid formers, others are neutral types, still others are strongly proteolytic forms.

This is followed by protein decomposition and putrefaction of the casein. In this condition toxic waste products may be present in the milk, rendering it not only unfit but dangerous for human consumption. Milk and cream are generally stored at 0°C. but the period at which it can be kept at this temperature should not be over 10 days for the above reasons.

The bacterial flora of milk kept at different temperatures is as follows:

0 to 5°C.—The fluorescent bacteria predominate. This includes *Pseudomonas fluorescens*, *P. schuylkilliensis*, *Bacillus fluorescens*, etc.

5 to 10°C.—Fluorescent bacteria (see above), *Proteus vulgaris*, micrococci such as *Micrococcus caseolyticus*, *M. flavus*, *M. conglomeratus*, *M. roseus*, *M. freudenreichii*, *M. epidermidis*, *M. candicans*, *M. viscosus*, etc., alkali-producing organisms including *Alcaligenes viscosus*, *A. marshallii*, *A. albus*, etc.

10 to 15°C.—Streptococci such as *Streptococcus agalactiae*, *S. lactis*, *S. cremoris*, *S. faecalis*, *S. liquefaciens*, *S. acidominimus*, etc., and *Aerobacter aerogenes*.

15 to 30°C.—Streptococci, especially *S. lactis.*

30 to 40°C.—*Escherichia coli, Aerobacter aerogenes,* the lactic acid-forming rods such as *Lactobacillus caucasicus, L. lactis, L. helveticus, L. bulgaricus, L. thermophilus, L. casei, L. plantarum, L. leichmannii, L. brevis, L. fermenti,* etc., and a few streptococci.

40 to 50°C.—Lactic acid-producing rods, including *Lactobacillus caucasicus, L. lactis, L. helveticus, L. bulgaricus, L. thermophilus,* etc., *Streptococcus faecalis, S. thermophilus,* yeasts, etc.

The smaller the initial plate count the greater will be the time required to sour the milk. As has already been shown, the fluorescent bacteria found in the soil are able to multiply at a temperature as low as 0°C. If milk is to be kept for any length of time it should be frozen; at somewhat higher temperatures organisms of the *Proteus* group develop, with the result that putrefactive products accumulate in the milk.

Coagulation seldom takes place in milk stored below 10°C. Above this temperature a coagulum forms in a few days owing to the combined action of rennin and acid-producing organisms. At a temperature of 20°C. the bacterial flora is composed of about 90 per cent streptococci. This results in a rapid coagulation of the milk. The acidity produced is sufficient to inhibit the growth of most other species of organisms likely to be present. Above 20°C. rod-shaped bacteria predominate, which are capable of producing still higher concentrations of lactic acid. Also, this is the most favorable temperature range for the growth of the butyric acid-producing anaerobes.

The aerobic organisms grow best near the surface of milk where there is an abundant supply of dissolved oxygen. The organisms predominating near the bottom include the anaerobes and *Streptococcus lactis.* This means that the spontaneous curdling of milk usually starts from the bottom.

PRESENCE OF MEMBERS OF THE ESCHERICHIA AND AEROBACTER GROUPS IN MILK

The presence of *E. coli* in milk is indicative of fecal pollution. Since *A. aerogenes* is found on hay, grains, and other food crops consumed by cows, its presence in milk does not necessarily indicate a fecal contamination. The two organisms do not have the same significance in judging the quality of milk. For this reason tests should be based on a separation of the two subdivisions of the colon group (see page 427).

Generally speaking, the greater the care observed in producing, collecting, and handling milk the smaller will be the number of colon organisms present. This same situation applies to the total number of bacteria in milk. Occasionally large numbers of *A. aerogenes* are present in infected udders. Under these conditions milk should be drawn aseptically from the udders of the cows to determine the source of the contamination.

Pasteurized milk is practically free from members of the *Escherichia-Aerobacter* group. If such organisms are still present after pasteurization, it means that either the milk was improperly treated or that it became recontaminated by the handler, or by the equipment, or by the final container.

The methods followed for the identification of the gas-forming organisms in milk are similar to those employed for the bacteriological examination of water (page 420).

SLIMY OR ROPY MILK

Milk on standing occasionally takes on a slimy or ropy consistency, in which state it may be pulled out into long threads. The ropiness may show great variation. Sometimes the change is very slight; at other times the ropy consistency may be so pronounced that the material can be drawn out into threads 3 ft. or more in length.

Several organisms are capable of producing this condition in milk. Probably the most important species is *Alcaligenes viscosus*. This organism produces its maximum amount of ropiness at a temperature of 18 to 20°C. The ropy condition is due to the formation of capsular material by the organisms. The slime is a carbohydrate gum, although some protein as well as carbohydrate appears to be necessary for its formation. A microscopic examination of the milk reveals the presence of individual cells embedded in a mass of capsular material.

A. viscosus is a small, short rod, sometimes almost spherical in shape. The cells are usually Gram-negative, although occasionally they may appear Gram-positive. The organism produces both a pellicle and ropiness in milk. The pellicle is the result of the aerobic character of the organisms. This explains why the ropiness is often noted in the cream layer only. The organism produces an alkaline reaction with no coagulation of the casein.

Some members of the *Escherichia-Aerobacter* group have caused the ropy condition of milk. Most strains are unable to do so but occasionally a strong capsule producer is encountered. *Aerobacter aerogenes* is probably of more frequent occurrence than *E. coli*.

Another organism producing ropiness in milk is *Micrococcus cremoris-viscosi*. This organism produces an acid coagulation of milk followed by a peptonization and the appearance of slimy material. It grows best at a temperature of about 30°C.

The source of the organisms causing ropy milk is the water used in the stable and dairy and also the coats of the cows. When once present, it is of utmost importance to remove the organisms as quickly as possible, otherwise great economic losses may result. All utensils and equipment coming in contact with the milk should be sterilized. The stable should

be thoroughly cleaned and disinfected. The flanks of the cows should be wiped with a cloth wetted with an appropriate disinfectant. The organisms causing ropiness are generally destroyed in one of the pasteurization processes, but outbreaks sometimes occur in pasteurized milk. These are due to contamination from the plant equipment after the pasteurization process.

Slime-producing, lactic acid organisms have been used in the manufacture of cheese, but such practice has been largely discontinued owing to the fact that it is difficult to separate the whey from the cheese. Also, the presence of the organisms in cream results in a poor yield of butter. The property of producing slime appears to be lost by growing the organisms at higher temperatures. Conversely, some organisms that ordinarily do not produce slime can be made to do so if cultivated at lower temperatures.

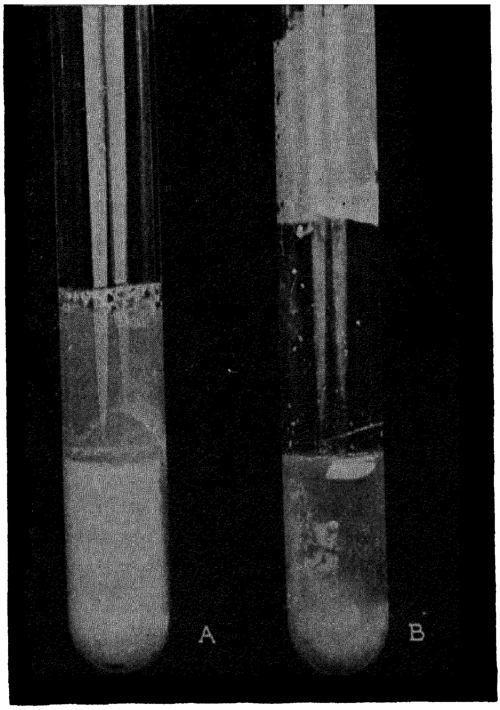


FIG. 162.—Stormy fermentation of milk. *A*, fermentation of a sample of milk free from *Clostridium welchii; B*, fermentation of a sample of milk containing *Cl. welchii.* The vaspar seal is pushed up by the gas pressure and the casein is curdled.

CLOSTRIDIUM PERFRINGENS IN MILK

Clostridium perfringens (*welchii*) is an anaerobic, spore-forming organism of widespread occurrence in nature. The organism is present in the intestinal tract of man and animals, in the soil, in fish, mollusks,

milk, cheese, water, etc. It occurs abundantly in the soil. The organism is generally considered the most important etiological factor in gas gangrene.

The organisms are short, thick rods occurring singly, in pairs, and less frequently in short chains. The rods are nonmotile, capsulated, and Gram-positive. The spores are large, oval, and central or subterminal.

It is a strongly saccharolytic organism being capable of fermenting all the common sugars with the production of acid and large amounts of gas. The organism produces a characteristic "stormy" fermentation of milk (Fig. 162). The curd becomes torn to shreds by the vigorous fermentation and evolution of gas. In this condition the milk proteins are not attacked.

The presence of *C. perfringens* in milk usually indicates a manurial contamination since the organisms are present in the intestinal contents of cows.

COLORED MILK

Several organisms have been isolated that are capable of changing the color of milk. These changes occur only occasionally and are of minor importance economically.

Many organisms isolated from milk produce colored colonies but these are not to be confused with those organisms which are capable of elaborating brilliant colors in milk. Chromogenic organisms are usually strongly aerobic. This means that growth and pigment formation are observed first in the surface layer of milk.

Blue Milk.—Blue milk is caused by the growth of the pigmented organism *Pseudomonas syncyanea.* This is a fluorescent organism and is capable of producing a gray color in neutral or alkaline milk. In the presence of acid the gray color is changed to blue. Therefore, acid-producing organisms, such as *Streptococcus lactis,* must be present to change the gray color to blue. The organism grows best at a temperature of about 25°C.

Red Milk.—The growth of *Serratia marcescens* produces a red color in milk. It is a small coccobacillus occurring singly and occasionally in short chains. The cells are motile and Gram negative. The organisms produce an acid reaction in milk with the formation of a soft coagulum. Since pigment production is best in the presence of an abundance of oxygen, the red color appears first on the surface of milk. The organism grows best at a temperature of about 25°C.

Yellow Milk.—[illegible] produces a canary yellow pigment when grown in milk. The organism produces a slow fermentation of the lactose, resulting in a coagulation of the casein. Since the slow fermentation fails to exhibit a protein-sparing action, the casein is

attacked and peptonized. The alkaline products of peptonization are sufficient to neutralize the primary acidity to give an alkaline, ropy milk. The organism grows best at a temperature of about 30°C.

REDUCTION OF METHYLENE BLUE

Methylene blue behaves like a respiratory pigment when introduced into bacterial cultures. It possesses the power of uniting with hydrogen, resulting in an intracellular oxidation. At the same time the dye is decolorized to the leuco compound. An aeration of the culture results in a loss of hydrogen followed by a restoration of the blue color. The decolorization of the dye is the result of the consumption of the dissolved oxygen by the growing bacteria. Therefore the methylene blue reduction time will depend upon the oxygen-consuming power of the organisms present (see page 272 for the reaction).

Methylene blue is of value in making a rapid survey of the quality of raw milk. The rate of decolorization depends upon the number of organisms present. The test can be employed to determine, in a rough way, the bacterial population of a milk sample. The procedure is quickly and easily carried out and with a minimum of expense. It is particularly valuable in making rapid inspections of large numbers of samples to determine if the milk received by companies answers the requirements prescribed by law.

The test is expressed as the period of time required for the color of methylene blue to disappear when incubated at 37°C. Under some conditions the blue color does not disappear uniformly. In such cases the end point is taken as the time required for the milk to show no blue color after it is mixed.

There is not always good agreement between the methylene blue reduction time and the agar plate count because (1) some organisms fail to grow on nutrient agar; (2) a clump of organisms records as only one colony whereas the rate of decolorization is due to the combined effect of each member of the mass; (3) the rate of decolorization of the dye is not the same for all organisms; and (4) the test becomes less accurate as the reduction time is increased, freshly drawn milk requiring at least 10 hr. to decolorize methylene blue. *Streptococcus lactis* reduces methylene blue more vigorously than any of the other organisms likely to be found in milk.

The classification of milk on the basis of the methylene blue reduction time is as follows:

Class 1.—Excellent milk, not decolorized in 8 hr.
Class 2.—Good milk, decolorized in less than 8 hr., but not less than 6 hr.
Class 3.—Fair milk, decolorized in less than 6 hr., but not less than 2 hr.
Class 4.—Poor milk, decolorized in less than 2 hr.

MILK-BORNE INFECTION

Milk is an excellent culture medium for a great variety of organisms. Pathogenic as well as saprophytic organisms not only remain viable for considerable periods but are capable of multiplying in milk. For this reason it is difficult to obtain pure milk and keep it pure. The prevention of milk-borne disease is one of the most important problems of public health.

Pathogenic organisms of both bovine and human origin have been isolated from milk. Many serious epidemics have been caused by the consumption of such products before this fact was clearly recognized. This is to be expected when one takes into consideration the enormous quantities of milk and its products that are consumed daily. Even today epidemics are spread through milk but they are of rare occurrence compared to the number reported during the early years of public health.

The abnormal changes that occur in milk are usually easily detected by appearance, taste, and smell. The presence of disease organisms in milk cannot be shown by such a procedure. Milk containing disease bacteria looks normal in appearance and gives no warning to the consumer. The disease organisms in milk may be derived (1) from diseased cows or (2) from the persons collecting and handling the milk.

Diseases of Bovine Origin.—The disease organisms of bovine origin found in milk include (1) *Mycobacterium tuberculosis* var. *bovis;* (2) *Streptococcus agalactiae;* (3) *Brucella abortus;* and (4) the virus of foot-and-mouth disease.

Tuberculosis is common among dairy cows. The organism of bovine tuberculosis is very similar in appearance to the species causing the disease in humans. Adults are probably not susceptible to infection by the organism causing bovine tuberculosis, but children, especially those under 5 years of age, may become infected by drinking raw milk from tuberculous cows. If the udders of cows are infected with the organisms, contamination of milk cannot be avoided. If cows are suffering from tuberculosis of the lungs, the sputum is swallowed, instead of being expectorated, with the result that the organisms appear in the feces. Since most milk contains some excreta, it is likely to show the presence of such organisms. It is doubtful if the organism multiplies in milk but it can live in milk and may retain its virulence for a considerable period of time.

Streptococcus agalactiae is found in infected udders and is responsible for the disease of cows known as mastitis. If the mastitis is severe, pus and blood may appear in the milk. Milk containing appreciable numbers of the organism, together with blood, must be regarded as unfit for human consumption.

Another disease organism frequently found in cows' milk is *Brucella abortus*. This organism produces contagious abortion in cows. The organism may produce the same effect in mares, sheep, rabbits, and guinea pigs. Organisms of a similar nature are *B. melitensis* from goats and *B. suis* from hogs. *B. melitensis* may also infect cows and be excreted in the milk. *B. suis* produces abortion in swine and frequently attacks horses, dogs, cows, monkeys, and laboratory animals.

All three of the organisms are pathogenic for man, producing the disease known as Malta fever, so named because it has been prevalent for centuries on the island of Malta in the Mediterranean where humans become infected by drinking contaminated goats' milk. The disease is now generally referred to as undulant fever or brucellosis (after Bruce who first isolated the organisms from the spleen in fatal cases of Malta fever).

Undulant fever may be contracted by drinking raw milk and, less frequently, certified milk. Pasteurized milk should be safe since the organisms are destroyed in the heat process. Because of this fact many public health authorities believe that all milk should be pasteurized before it reaches the consumer.

Foot-and-mouth disease is a highly contagious virus disease of domesticated animals. It produces fever, digestive disturbances, and a vesicular eruption on the mucous membranes of the mouth and on the skin between the toes. The vesicles may be present also on the udder and teats of the cow. The virus produces a high death rate among cattle. In man the death rate is low, owing to the fact that the disease runs a mild course.

The presence of the virus in infected cattle may be demonstrated in milk, saliva, urine, and feces. The infection may be transmitted by feeding and drinking troughs, stalls, cattle cars, etc. The disease is stamped out by slaughtering herds showing the presence of any infected animals.

Diseases of Human Origin.—Some of the diseases of human origin that have been disseminated by milk are (1) typhoid fever, (2) scarlet fever, (3) diphtheria, (4) septic sore throat, (5) infantile diarrhea, and (6) infantile paralysis. The organisms may be transferred to milk by contaminated hands of the workers, by droplets expelled during coughing, sneezing, and talking, by moistening the hands with saliva during wet milking, and in other ways.

Many typhoid epidemics that have occurred in recent years have been traced to the consumption of contaminated milk. Further investigation usually revealed the fact that only one dairy supplying the milk was responsible for the spread of the infection. The organisms were introduced into the milk by a typhoid carrier or an unrecognized case of typhoid fever among the workers at the dairy. The isolation of this

individual resulted in a disappearance of new typhoid cases in the community. Typhoid epidemics have been traced not only to milk but to a lesser extent to ice cream, cheese, and butter.

Scarlet fever and septic sore throat are probably both caused by *Streptococcus pyogenes*, a pus-producing organism. Epidemics have been caused by the consumption of milk containing this organism. The milk may become contaminated by handlers or by infected udders of cows. Usually a milker suffering from scarlet fever or sore throat infects the udders with the organisms by means of his contaminated hands. The organisms rapidly multiply in the milk left in the udders. Abscesses form in the udders from which the milk becomes heavily contaminated. The contaminated milk may produce septic sore throat or scarlet fever in persons who consume the raw milk.

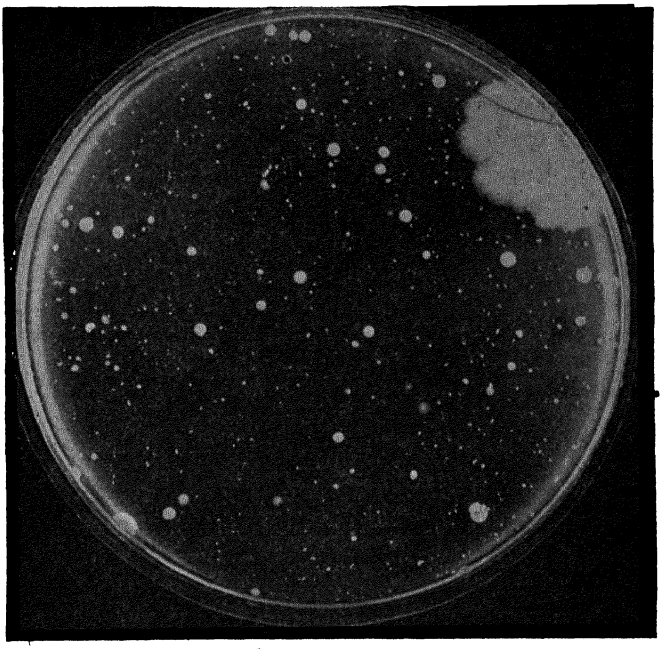
PASTEURIZATION OF MILK

The destruction of all organisms in milk is called sterilization. The high temperature required for this purpose results in milk with a cooked flavor. Such milk possesses two serious objections: (1) the cooked flavor is not so pleasant as that of raw milk and (2) heating to a high temperature may result in a decrease of the vitamin content. These objections are overcome for the most part by heating milk to temperatures lower than that required to sterilize completely but sufficiently high to destroy all disease organisms.

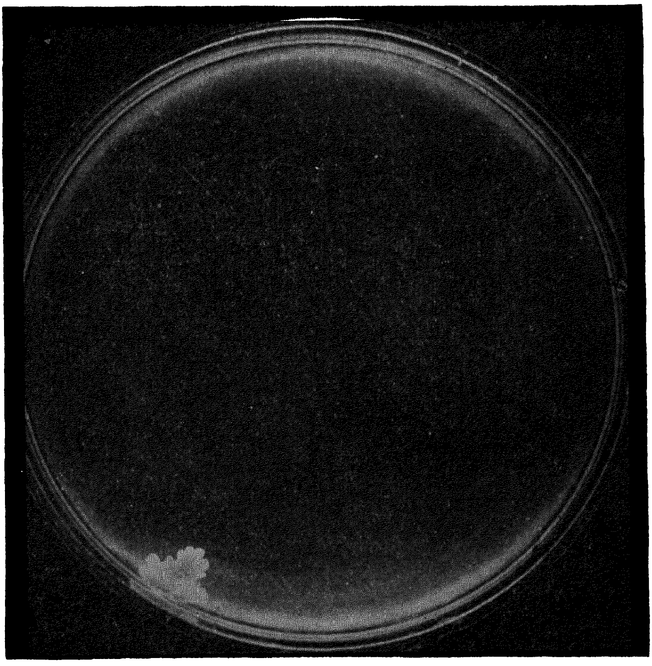
The destruction of disease organisms in milk without resorting to complete sterilization is called pasteurization. Two methods are employed for the commercial pasteurization of milk: (1) the flash method and (2) the holder method. In the flash method the milk is heated to a temperature of 80°C. for 2 min., then quickly cooled to prevent the milk from acquiring a cooked flavor. The same result may be obtained by lowering the temperature and prolonging the exposure time. Accordingly, the holder method makes use of a temperature of 60 to 65°C. for 30 min., after which the milk is quickly cooled. In the United States the holder method is used to a greater extent than the flash method.

Low-temperature pasteurization has a less destructive effect on the proteins of milk than the flash method and is recommended for milk that is to be used for infant feeding and for cheese making. Orla-Jensen (1931) showed that when milk was heated to temperatures above 70°C. the coagulation time was decreased and the time required to coagulate with rennin was increased.

During pasteurization the milk must be kept in constant agitation to prevent the formation of a scum on the surface. The presence of a scum results in the formation of a protective layer around many of the bacteria, which prevents the penetration of heat. The reaction of milk should be



A



B

Fig. 163.—Bacterial count of milk. *A*, raw milk; *B*, same milk after pasteurization.

as nearly neutral as possible. If it is acid, a coagulation of the casein may occur during the heating process.

The pasteurization process reduces the bacterial count from 90 to 100 per cent (Fig. 163). The percentage varies, depending upon the kinds and numbers of organisms present at the time of heating. The lactic acid-producing bacteria are more resistant to heat than the usual, nonspore-forming disease organisms. Many of them are not destroyed by either low- or high-temperature pasteurization. This explains why milk quickly sours unless it is stored at a low temperature.

BUTTER

Butter is composed of milk fat, water, casein, lactose (milk sugar), and salt. The water content varies from 10 to 16 per cent by weight. The amount of casein and lactose present depends upon the extent to which butter is washed during the process of manufacture. From 1 to 3 per cent of salt is usually added, which is completely dissolved in the water. Since the salt does not dissolve in the butter fat, the liquid portion of butter consists of a 10 to 30 per cent solution of salt.

Butter was originally prepared by churning fresh, sweet cream, either raw or pasteurized, in order to separate the fat globules from the other constituents of cream. This method is no longer practiced to any great extent because it necessitates churning daily while the milk or cream is still fresh. The tendency now is to allow the cream to sour first, after which the fat may be more easily separated from the casein.

The cream may be soured naturally or by the addition of a culture of lactic acid organisms known as a starter. The advantages of first souring the cream are (1) the yield of butter is increased, owing to a better separation of the casein and fat, and (2) the aroma and flavor may be greatly improved.

If fresh sweet cream is used, the butter will have a mild flavor consisting chiefly of the natural flavor of the milk fat. If the cream is carelessly handled and not cooled at a sufficiently low temperature, the organisms will multiply at a very rapid rate. The resulting aroma and flavor will depend largely on the organisms predominating in the cream. Cream contains a low content of casein and lactose. This means that the flavor and aroma are likely to be undesirable because the acidity produced will be insufficient to suppress the growth of the putrefactive organisms.

Butter Cultures or Starters.—In order to overcome the development of undesirable flavors, cream is usually pasteurized to destroy the organisms present and then allowed to sour by the addition of cultures or starters of lactic acid bacteria. The organisms selected are those known to produce desirable flavors and aromas. The organisms are allowed to

grow in cream until the desired hydrogen-ion concentration is reached. The cream is then churned to separate the butter fat. The lactic acid bacteria tend to inhibit the growth of other organisms. The result is that butter prepared in this manner has better keeping qualities than that made from raw, unpasteurized cream, which may be heavily contaminated with organisms likely to produce undesirable flavors and aromas. Another great advantage to the use of cultures or starters is that a uniform product may be obtained with comparative ease.

Organisms in Starters.—Several species of organisms have been used as starters. The organisms to be employed depend largely upon the aroma and flavor desired in the finished product. Some of the organisms used for this purpose are (1) *Streptococcus thermophilus*, (2) *S. cremoris*, (3) *S. lactis*, (4) *Lactobacillus helveticus*, (5) *L. bulgaricus*, (6) *Leuconostoc dextranicum*, and (7) *L. citrovorum*.

Streptococcus thermophilus.—These organisms are spheres with pointed ends, and occur singly and in short chains. They are Gram-positive and grow actively at a temperature of 50°C. *S. thermophilus* is not destroyed at temperatures of 60 to 65°C. for 30 min., being capable of resisting the pasteurization treatment. The organism is very fastidious in its nutritive requirements. It is not very viable on the usual laboratory media. On nutrient agar it produces small, pin-point colonies. It ferments the lactose of milk with the production chiefly of lactic acid. *S. thermophilus* is extremely sensitive to salt, no growth occurring in a 2 per cent solution. The optimum growth temperature is 50°C. The final pH in broth cultures is from 4.5 to 4.0. It has been isolated from milk and milk products and is used as a starter in the preparation of Swiss cheese.

Streptococcus cremoris.—This organism is, as a rule, larger than *S. lactis*. It forms long chains in milk. The cells are Gram-positive. *S. cremoris* ferments the lactose of milk with the production chiefly of lactic acid. The optimum growth temperature is about 30°C. Its thermal death point is 65 to 70°C. for 10 min. The organism grows in a 2 per cent but not in a 4 per cent salt solution. It grows poorly on artificial media. *S. cremoris* has been isolated from raw milk and milk products. It is commonly employed in commercial starters for the manufacture of butter and cheese.

Streptococcus lactis.—The characteristics of this organism are given on page 439.

Lactobacillus helveticus.—The organisms are large nonmotile rods, occurring singly and in chains. They are Gram-positive. The lactose of the milk is fermented with the production chiefly of lactic acid. *L. helveticus* grows best at a temperature of 40 to 42°C. It was originally isolated from dairy products and is commonly employed as a starter in the preparation of butter and cheese.

Lactobacillus bulgaricus.—This species is also composed of large, nonmotile, Gram-positive rods, which may appear singly and in chains. It ferments lactose vigorously with the production of lactic acid and other compounds. The organism grows best at a temperature of 45 to 50°C. *L. bulgaricus* was originally isolated from yoghurt and is present in many milk products. It is commonly employed as a starter in the manufacture of butter and cheese.

Leuconostoc dextranicum.—Cultures of this organism are composed of spherical cells, which occur in pairs and in short chains. The cells are Gram-positive. The organism grows best at 21 to 25°C. *L. dextranicum* occurs in plant materials and in milk products. It is frequently employed in dairy starters.

Leuconostoc citrovorum.—This organism is similar to *L. dextranicum.* The cells are spheres, which occur in pairs and in chains. The organisms are Gram-positive. *L. citrovorum* grows best at 20 to 25°C. It utilizes citric acid in milk. The organism has been isolated from milk and dairy products and is used as a starter in the manufacture of butter.

At the present time the starters that appear to be used more than any of the others are those containing the two organisms *Leuconostoc dextranicum* and *L. citrovorum.* The former ferments lactose with the formation of lactic acid, whereas the latter utilizes the citric acid in the milk with the liberation of various compounds. These organisms impart very desirable aromas and flavors to butter, cheese, and fermented milk.

van Niel, Kluyver, and Derx (1929), Schmalfuss and Barthmeyer (1929), and others showed that the aroma and flavor imparted to butter by the growth of these two organisms was due to the presence of the compounds acetylmethylcarbinol ($CH_3 \cdot CO \cdot CHOH \cdot CH_3$) and diacetyl ($CH_3 \cdot CO \cdot OC \cdot CH_3$). Diacetyl in high dilution suggests the odor of butter. Acetylmethylcarbinol in pure form is odorless but in the impure state it gives off an odor not unlike that of diacetyl. Later Michaelian, Farmer, and Hammer (1933) found that cultures having a satisfactory aroma and flavor contained relatively large amounts of these two compounds whereas those cultures which did not have a satisfactory aroma and flavor contained deficient amounts of the compounds.

Leuconostoc citrovorum (and possibly *L. dextranicum*) ferments citric acid with the formation of acetic acid, diacetyl, acetylmethylcarbinol, 2:3-butylene glycol, and carbon dioxide. Pure cultures in milk yield acetic acid, 2:3-butylene glycol ($CH_3 \cdot CHOH \cdot CHOH \cdot CH_3$), and carbon dioxide but when the pH is lowered to a certain point acetylmethylcarbinol and diacetyl accumulate in the culture. Under favorable conditions some acetylmethylcarbinol is reduced to 2:3-butylene glycol but when the conditions are unfavorable the reduction is so slow that

acetylmethylcarbinol accumulates in the culture and is slowly oxidized to diacetyl. The addition of citric acid to the medium greatly increases the amounts of the various compounds produced.

The first compound produced is believed to be acetylmethylcarbinol. This is oxidized to diacetyl. Under favorable conditions, especially a low acidity and suitable temperature, some acetylmethylcarbinol is reduced to 2:3-butylene glycol. According to Rumments (1936) the reactions are as follows:

$$2\underset{\text{Citric acid}}{\begin{matrix} CH_2{\cdot}COOH \\ | \\ COH{\cdot}COOH \\ | \\ CH_2{\cdot}COOH \end{matrix}} \rightarrow \underset{\text{Acetylmethyl-carbinol}}{CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3} + \underset{\text{Acetic acid}}{2CH_3{\cdot}COOH} + 4CO_2 + H_2O$$

$$\underset{\text{2:3-butylene glycol}}{CH_3{\cdot}CHOH{\cdot}CHOH{\cdot}CH_3} \xleftarrow{\text{Reduction}} 2H + \underset{\text{Acetylmethyl-carbinol}}{CH_3{\cdot}CO{\cdot}CHOH{\cdot}CH_3} \xrightarrow{\text{Oxidation}} \underset{\text{Diacetyl}}{CH_3{\cdot}CO{\cdot}OC{\cdot}CH_3} + 2H$$

Preparation and Use of Starter.—The starter is prepared by pasteurizing milk at a high temperature, *i.e.*, 75 to 95°C. for 15 min., then inoculating it with the organisms. After the starter is prepared, it should be handled with great care to prevent as far as possible the entrance of organisms that are likely to produce undesirable fermentations. It is much better to add pure cultures of the organisms to the cream rather than to run the risk of an abnormal fermentation by the use of a starter.

The amount of starter to be added varies considerably, depending upon the temperature of incubation and other factors. Usually from 0.25 to 1 per cent of starter is added to cream that is to be incubated at a temperature of 18 to 20°C.

Undesirable Changes in Butter.—Butter prepared from sweet, unpasteurized cream contains the same microflora as the cream from which it was prepared. Also, the bacterial changes that take place during storage are the same as those which occur in milk kept under the same conditions. Butter prepared from cream previously pasteurized at high temperatures, and then inoculated, generally contains only those organisms that were added to promote ripening. Some molds and yeasts may be present, which result from air contamination. Since molds are strongly aerobic, they grow only on the surface of butter.

Undesirable changes that take place after butter has been manufactured are due largely to the activities of microorganisms. Many of the organisms responsible for producing defects in butter are present as a result of contamination after its manufacture. Therefore, the same precautions used in handling milk and cream apply in the handling of butter. The extent of recontamination is roughly an indication of the care exercised in handling the butter.

Rancidity of Butter.—The first stage in the appearance of rancidity is a hydrolysis of the glycerides, comprising the fat, to glycerol and fatty acids. Many organisms, both aerobic and anaerobic, have been shown to be capable of producing the reaction.

Fishiness in Butter.—Fishiness in butter is due to high acidity and to the decomposition of lecithin resulting in the formation of trimethylamine. Lecithin is composed of fatty acids, glycerol, phosphoric acid, and choline (see page 241). Trimethylamine originates from the base choline. The organism *Proteus ichthyosmius*, first isolated from a can of evaporated milk having a fishy odor, is capable of producing the same effect when inoculated into fresh milk. The organism is a Gram-negative, motile rod, occurring as single cells. It does not ferment lactose. The optimum temperature for growth is 20°C.

Tallowiness in Butter.—A tallow-like odor is due largely to oxidation. This may result from the action of the ultraviolet rays of sunlight or of the oxidases naturally present in cream. It has been said that certain mold enzymes are capable of producing a similar effect. The changes may be prevented by high-temperature pasteurization, which results in a destruction of the oxidizing enzymes.

Quantitative Bacteriological Examination of Butter.—Butter is not a favorable medium for the growth of most bacteria. This means that multiplication usually occurs only in the small drops of water containing the dissolved salt and possibly some lactose and casein. Since the high-salt content makes this an unfavorable medium, butter never shows bacterial counts so high as those obtained from milk. The count is highest in freshly prepared butter and becomes less and less as the butter ages.

The bacterial flora of the surface of butter differs from that of the interior, owing to contamination from the air and difference in the oxygen tension. For these reasons it is difficult to obtain a representative sample for examination. A sample is usually obtained by removing a cylinder of butter from a cake by means of a sterile sampler. The butter is melted in a water bath at a temperature not to exceed 40°C. and dilutions prepared in sterile water blanks, previously heated to the same temperature.

ICE CREAM

Ice cream is a frozen dairy product composed of cream, sugar, gelatin, and flavoring. Sometimes condensed milk is also added. Unlike the preparation of butter and cheese, bacteria play no part in the process of manufacture.

The bacterial content of ice cream depends largely upon (1) the number present in the cream at the time of preparation and (2) the

number of organisms present in the various ingredients employed in its manufacture. It has been shown that some bacteria actually multiply in ice cream kept in cold storage. The numbers decrease at first, then show a gradual increase after a period of about a month.

The lactic acid organisms, *i.e.*, those which are responsible for the souring of cream, fail to multiply at low temperatures. An increase in numbers indicates the presence of other species, such as the putrefactive types. These organisms may possibly produce objectionable metabolic waste products. The presence of pathogenic organisms in ice cream is usually the result of using contaminated cream in its manufacture.

CHEESE

According to the standards of the Food and Drug Administration of the U.S. Department of Agriculture, cheese may be defined, as,

> . . . the product made from the separated curd obtained by coagulating the casein of milk, skimmed milk, or milk enriched with cream. The coagulation is accomplished by means of rennet or other suitable enzyme, lactic fermentation, or by a combination of the two. The curd may be modified by heat, pressure, ripening ferments, special molds, or suitable seasoning.

The solid curd is molded into various shapes according to the variety of cheese being manufactured. Freshly prepared and molded curd is known as green cheese. In order that it be made satisfactory for consumption it must be set aside to ripen. Certain conditions, such as temperature and moisture, are carefully controlled during the ripening process. The cheese changes considerably during this stage. The insoluble casein is rendered soluble and the digestibility is greatly improved. The consistency changes, resulting in a softer product. Also, the flavor characteristic of the finished product develops during the ripening period.

Two general processes are followed for the preparation of curd. One is due to the action of enzymes; the other is associated with the growth of organisms. The latter is indirectly also an enzymatic process. The cheeses in the first group are known as rennet curd cheeses whereas those in the second group as acid curd cheeses.

As has already been said, the changes that take place during ripening are largely enzymatic. However, this does not explain all the changes that occur during the long aging period. The flavors that make their appearance during the latter period of the ripening process are not the result of enzymatic action but of the associated activities of bacteria, yeasts, and molds. The enzymes improve the consistency and digestibility of cheeses but play no part in improving the flavors. The flavors

and characteristics of the various cheeses depend upon the kinds and numbers of organisms present.

Hard Cheeses.—Hard cheeses are prepared from curd subjected to heavy pressure to remove as much of the whey as possible. This gives a very hard, tough curd, which does not become softened to any extent during the ripening period. Examples of hard cheeses are American (Cheddar) cheese, Swiss cheese, Cheshire cheese, and Edam cheese. Since the curd is very compact and tough, the ripening stage requires a considerable period of time to produce a satisfactory product. Enzymatic and bacterial changes proceed simultaneously.

Several phases may be recognized during the ripening period. In the first phase the lactic acid bacteria multiply more rapidly than the other species present, resulting in a predominance of these organisms. This continues for several days, after which the numbers show a gradual decrease. At the end of the ripening period the number of lactic acid bacteria may be smaller than at the beginning of the process.

Desirable Organisms.—Many organisms are responsible for the aromas, flavors, and characteristics of the various types of cheeses. Apparently each type has its own characteristic flora. Some of the organisms that have been isolated from cheeses are (1) *Streptococcus lactis* (page 439), (2) *S. cremoris* (page 455), (3) *Leuconostoc citrovorum* (page 456), (4) *L. dextranicum* (page 456), (5) *S. thermophilus* (page 455), (6) *Lactobacillus casei*, nonmotile, Gram-positive rods, ferment lactose with the formation chiefly of lactic acid, optimum temperature 30°C., (7) *L. lactis*, rods occurring singly, in pairs, and chains, Gram-positive, produce lactic acid from lactose, optimum temperature 40°C., (8) *L. bulgaricus* (page 456), (9) *L. helveticus* (page 455), (10) *L. plantarum*, large rods, occurring singly and in short chains, nonmotile and Gram-positive, lactic acid produced from lactose, optimum temperature 30°C., (11) *Propionibacterium shermanii*, small spherical cells, mostly in pairs and short chains, anaerobic, nonmotile, and Gram-positive, produce lactic acid from lactose.

Undesirable Organisms.—The presence of undesirable organisms is responsible for numerous types of faulty cheeses. The milk becomes contaminated through carelessness in collecting and handling. Considerable losses are experienced at times by cheese manufacturers. For this reason it is generally advisable to use milk previously pasteurized and then inoculated with the desired organism or organisms rather than to start with unpasteurized or raw milk. Some cheese faults affect the taste, others are concerned with the appearance of the finished product. One of the most common faults is swelled or blown cheese, due to fermentation of lactose with the production of acid and gas. The gas bubbles cause the cheese to swell until it may actually burst. Unpleasant

flavors are produced by the organisms. The members of the *Escherichia-Aerobacter* group are usually involved.

The presence of putrefactive organisms may be responsible for putrid odors and flavors. The bacteria proliferate and become active when the acidity of the cheese is reduced during the ripening period. The presence of chromogenic organisms may be responsible for discolorations in cheese. This may be due also to various chemical reactions with metals, such as copper and iron, from utensils used in handling the raw materials. Blue spots in Edam cheese are said to be due to *Bacillus cyaneofuscus.* The organisms die before the cheese has fully ripened. Red and brown spots in Emmental cheese are caused by the growth of chromogenic propionic acid bacteria. *Lactobacillus brevis* var. *rudensis* is responsible for the appearance of rusty spots in Cheddar cheese. Since it is particularly prevalent in the spring, the organism is believed to originate from green grass. Surface discolorations are produced by many molds such as *Penicillium casei, Cladosporium herbarum, Monilia niger,* and *Oospora crustacea.* Red and yellow torulae (false yeasts) play some part in the process.

Soft Cheeses.—Soft cheeses are prepared by allowing the whey to drain from the curd without the application of pressure. Cheeses prepared in this manner contain more moisture than hard curd cheeses and result in a much softer finished product. The first stage in the preparation of soft cheeses is a fermentation of the lactose by means of lactic acid bacteria with the formation of an acid curd. Subsequent changes depend upon the control of certain well-defined conditions.

In the Camembert types the surface of the cheese is kept dry. This furnishes an excellent opportunity for the growth of certain molds such as *Penicillium camemberti* and *Oidium lactis.* Enzymes secreted by *P. camemberti* act upon the surface of the cheese to produce a slow liquefaction of the casein. The enzymes gradually penetrate the product until the whole of the curd is affected. The result is the formation of a soft, creamy mass at the completion of the ripening period. The characteristic flavor is due probably to the growth of *O. lactis.*

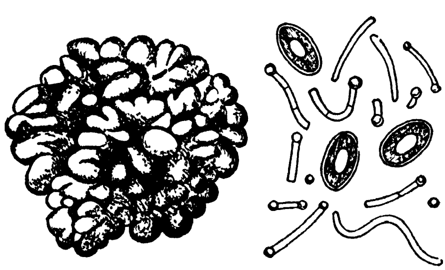
In cheeses of the Roquefort type the blue-green mold *P. roqueforti* and related species are inoculated into the curd. Since the organisms are aerobic, holes are punched in the curd to facilitate development of the mold throughout the cheese. The enzymes elaborated by the organisms soften the casein and certain metabolic products produce the characteristic aromas and flavors.

Cheeses of the Limburger type are produced by keeping the surface wet to prevent the growth of molds and to stimulate the growth of bacteria. The ripening process involves the decomposition of casein by enzymatic and bacterial action.

FERMENTED MILK

Milk is probably the most important article of food among many pastoral tribes of Europe and Asia. Because of the primitive sanitary conditions under which the people live, the milk is usually fermented before it is consumed. This is especially true during the warm seasons of the year.

The consumption of soured milk preparations is widespread because of their supposedly therapeutic value. The fact that they appear under various names does not mean necessarily that each product is fermented with a different organism. The names identify the country or region in which they are produced. Many of these preparations result from the combined action of two or more organisms. They furnish excellent illustrations of associations.



A B

FIG. 164.—*A*, Kefir grain. *B*, yeasts and bacterial cells present in the grains. (*After Freudenreich; from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

Yogurt.—The soured milk preparation of Bulgaria and Turkey is known as yogurt. This is usually prepared from camels' or mares' milk. The fermentation is produced by acid-forming organisms of the *Lactobacillus bulgaricus* type. Sometimes yeasts are also present and produce a small amount of alcohol and carbon dioxide. The organisms produce from 1.5 to 2.5 per cent acid calculated as lactic acid.

Matzoon.—This is the soured milk preparation of Armenia and is similar to yogurt in flavor and microflora.

Gioddu.—Gioddu is the fermented milk preparation prepared on the island of Sardinia. It contains the same organisms as Bulgarian yogurt and Armenian matzoon.

Leben.—The Egyptian drink known as leben is prepared by the action of lactic acid-producing bacteria and yeasts on cows', goats', or buffalos' milk. The bacteria hydrolyze the lactose to glucose and galactose, after which some of the sugar is fermented by the yeasts to alcohol and carbon dioxide, and some attacked by the bacteria with the production of lactic acid.

Kumiss.—Kumiss is a Russian product prepared by the fermentation of mares' or cows' milk by yeasts, lactobacilli, and lactic streptococci. The yeasts produce alcohol and carbon dioxide, and the bacteria produce lactic acid.

Kefir.—Kefir is prepared by inoculating milk with kefir grains. These grains resemble cauliflowers in appearance (Fig. 164) and are composed

of tangled filaments of a yeast known as *Saccharomyces kefir*, *Lactobacillus casei*, and streptococci. The yeast produces alcohol and carbon dioxide, and the bacteria ferment the sugar to lactic acid.

Curds.—The fermented milk preparation of Ceylon is usually referred to as curds. It is manufactured from cows' or buffalos' milk. The milk is boiled, cooled, and, while still warm, inoculated with a piece of curd from a previous lot. The milk is allowed to ferment for at least 36 hr. before it is eaten. The organisms responsible for the reaction include yeasts, *Streptococcus lactis*, and a Gram-negative lactobacillus. In most

FIG. 165.—*Lactobacillus acidophilus* grown in soybean milk.

samples the yeast and *S. lactis* predominate. The preparation is similar to kumiss and kefir.

Acidophilus Milk.—Yogurt is consumed in large quantities as an article of diet by the people of Bulgaria. It has been noted that centenarians are more numerous in Bulgaria, in proportion to population, than in other countries. Metchnikoff claimed that the increase in the life span was due to the ingestion of large quantities of soured milk, produced by the action of the rod-shaped organism *Lactobacillus bulgaricus* (page 456). Because of this fact he advocated the consumption of soured milk for the prolongation of life.

According to Metchnikoff, growth of *L. bulgaricus* in the intestinal canal produced a high percentage of lactic acid, which was capable of

inhibiting the growth of the putrefactive bacteria. Disorders that were supposed to be associated with auto-intoxication (absorption of putrefactive metabolic waste products from the intestinal tract) would be prevented.

More recently it has been shown that *L. bulgaricus* is not a natural inhabitant of the intestinal tract of man and, therefore, does not become acclimated to the new environment. The consumption of Bulgarian milk stimulated the growth of the lactic acid rod organisms normally present in the intestinal tract. One of these organisms is *L. acidophilus* (Fig. 165).

L. acidophilus is present in the intestinal contents of adults. On a mixed diet, the numbers are small. If the diet is supplemented with large quantities of milk or carbohydrates, such as lactose and dextrin, *L. acidophilus* produces a high concentration of lactic acid (about 3 per cent), which is sufficient to inhibit the growth of the putrefactive organisms (*E. coli*, etc.). In the absence of a high carbohydrate diet the flora again becomes predominantly putrefactive in character.

For additional reading on milk and milk products consult the books and articles by Breed (1928), Burdon (1939), Eckles, Combs, and Macy (1936), Hammer (1938), Kopeloff (1926), Nicholls, Nimalasuriya, and De Silva (1939), Orla-Jensen (1931), Prescott and Dunn (1940), Prescott and Proctor (1937), Rettger, Levy, Weinstein, and Weiss (1935), Sherman (1937), and Wilson (1935).

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CHAPTER XXII

BACTERIOLOGY OF FOOD

Food may be preserved indefinitely if kept free from organisms, or if the organisms are prevented from multiplying. Decomposition and spoilage of food are the result of the activities of living organisms, particularly those grouped with the bacteria, the yeasts, and the molds.

Different kinds of organisms produce different types of changes in food. The decomposition of foods rich in carbohydrates results usually in various types of fermentations. The action of organisms on high-protein foods results in putrefactions. The products of the former are usually harmless, whereas those of the latter are objectionable and even dangerous.

Bacteria are more exacting in their requirements than either the yeasts or the molds. This means that yeasts and molds can multiply under conditions unfavorable to the growth of bacteria. Bacteria require relatively large amounts of moisture, hydrogen-ion concentrations usually near the neutral point, and relatively low osmotic pressures, for growth and multiplication. Yeasts can tolerate less moisture, are less exacting in their pH requirements, and can multiply in solutions having higher osmotic pressures. Molds are the least exacting of the fungi. They can withstand relatively high acidities, require far less moisture, even grow on substances almost dry, and can tolerate extremely high osmotic pressures.

PRESERVATION OF FOODS

The methods used at the present time were employed long before their modes of action were clearly understood. As their mechanisms became known various improvements were made. The methods commonly employed for the preservation of foods involve the use of (1) heat, (2) cold, (3) drying, (4) preservatives, and (5) high osmotic pressures.

Heat.—The use of heat is the method employed in home and commercial canning of meats, fruits, and vegetables. Heat is employed to effect either a complete sterilization or a reduction in the number of organisms that may be present. In the latter case the organisms that have not been killed are prevented from multiplying. Excessive heat is efficient in destroying all forms of microscopic life. The destructive action of heat is due probably to the coagulation of the protoplasm of living cells, rendering it incapable of carrying on its vital functions. It

is not desirable greatly to exceed the minimum temperature required to effect sterilization, otherwise alterations may occur in the appearance, flavor, and composition of foods. Since all bacteria are not necessarily killed, the term processing is generally employed in referring to heat-treated canned foods.

The various details of canning procedures necessarily vary with the nature of the product to be preserved. There are certain important operations common to all canning procedures: (1) the cleansing operation, (2) blanching, (3) exhausting or preheating, (4) sealing the tin container, (5) heat-processing the sealed container, and (6) cooling the tin container after thermal processing.

Cleansing Operation.—The first and one of the most important steps in commercial canning is the thorough cleansing of the food materials to be preserved. Cleansing serves two purposes. It makes a better looking product, and it serves to reduce substantially the load of spoilage bacteria, which may place a heavy burden on the heat process.

Cleansing may be effected by various types of washers. The raw materials are subjected to high-pressure sprays or strong-flowing streams of water while passing along a moving belt or while being dropped in agitating or revolving screens. With certain food materials dirt and other large, adhering particles are mechanically removed by means of revolving or agitating screens or by strong blasts of air.

Blanching.—The blanch involves the immersion of raw food materials, (fruits and vegetables) into warm or hot water, or exposure to live steam. This is practiced for several reasons. Blanching may serve only as a hot-water wash where adhering materials cannot be removed with cold water. It may soften fibrous plant tissue so that it will either contract (lose water) or expand (take up water). This ensures proper filling of the container. During the blanching operation respiratory gases are expelled. This prevents strain on the can during processing and favors the development of a higher vacuum in the finished product. Blanching inhibits the action of respiratory enzymes, especially those of oxidation, to give a product of superior quality and nutritive value. Lastly, blanching fixes the natural color of certain products and makes them more attractive in appearance.

Exhausting or Preheating.—All canning procedures provide for the exclusion of as much oxygen (air) as possible. The presence of oxygen is undesirable for two reasons. It may react with the food material and the interior of the container and affect the quality and nutritive value of the food. The presence of oxygen and other gases may cause undue strain on the container during the processing period.

The procedure followed in expelling gases consists in passing the open can, containing the raw food, through an exhaust box in which hot water

or steam is used to expand the food and expel air and other gases from the contents and the head-space area of the can. After the gases are expelled, the can is immediately sealed, heat-processed, and cooled. During the cooling, the contents of the can contract, creating a vacuum. This is accepted as evidence of soundness of the canned product.

Sealing the Tin Container.—Each can must be properly sealed before being subjected to the heat process. The heat destroys any organisms present in the raw food material and the seal on the can prevents reinfection of the contents. The sealing operation is, therefore, one of the most important steps in the canning procedure.

Heat-processing the Sealed Container.—The processing operation usually involves the application of steam under pressure (autoclave). This destroys pathogenic and other organisms capable of causing spoilage of the contents. The seal on the can prevents the contents from becoming recontaminated by the same or other kinds of organisms.

The time required for processing canned foods depends upon various factors, such as character and composition of the food, types and numbers of organisms likely to be present, and hydrogen-ion concentration of the food. Heat penetrates to the center of cans by conduction and convection. Penetration of solid foods by heat takes place by conduction and is relatively slow. Penetration of liquid foods takes place by conduction and convection, with the result that the action is more rapid. The size of the food particles also influences the speed of penetration by heat. The larger the particles the slower the penetration.

Bacteria are usually more easily killed in an acid or an alkaline environment than in a neutral one. Fruits and vegetables are, therefore, more easily processed than fish and meats. Also, fruits and vegetables are more easily penetrated by heat than are meats and fish. The temperature and time of processing must be determined for each kind of food. In general, nonspore-forming organisms in a liquid medium are destroyed at a temperature of 60°C. for 1 hr. or at 70 to 80°C. in a few minutes. Spores are not destroyed when subjected to the above temperatures. A temperature of 115°C. for 30 min. or 120°C. for 15 min. will usually destroy all forms of life.

In the processing of foods an excessive period of heating is avoided to prevent injury to the product. A long exposure at a relatively low temperature is usually preferable to a short exposure at a higher temperature. This applies especially to canned fruits.

Cooling the Tin Container after Thermal Processing.—The last operation in the commercial process involves rapid cooling of the sealed cans. This is necessary in order to check the action of the heat and prevent undue softening or change in color of the contents. The cans may be cooled by means of air or of water.

Air cooling is accomplished in well-ventilated, specially designed, storage rooms where the cans are stacked in rows with ample space for efficient circulation of air. Water cooling is accomplished by allowing water to run into the autoclave in which the cans are processed, or the cans may be removed from the sterilizer and conveyed through tanks of cold water or through cold-water showers.

Cold.—Two methods are followed in the preservation of foods by cold temperatures: (1) cold storage and (2) chilling. Both methods have their advantages and disadvantages.

In the cold-storage method the temperature is kept well below the freezing point. The food is frozen solid, producing some changes in its physical condition. Meats, fish, and poultry are usually preserved in this manner for long periods of time.

In the cold-storage process the food is first frozen in cold air or by immersion in salt solution. In the air method the food is held at a temperature of about $-15°C$. A long time is required in freezing the food and considerable moisture is lost by evaporation. In the brine process the food is immersed in salt solutions of different strengths. The addition of salt to water lowers the temperature at which freezing takes place. An increase in the salt concentration causes a lowering of the freezing point of the solution. A salt concentration of 25 to 28 per cent lowers the freezing point to about $-15°C$. The brine process has an advantage over the air method in that freezing takes place in a shorter period of time. An important disadvantage is that some substances are likely to diffuse out of the food and be replaced by salt.

After the food is frozen by either of the two methods, it is stored in a chamber where the air is kept below 0°C. Certain changes are known to take place during the storage period. Some water is lost by evaporation. Aromatic substances that impart flavor and odor to the food may disappear entirely. Fats and oils may be slowly hydrolyzed to glycerol and fatty acids. Microorganisms may slowly multiply on the surface of the food. The extent of these changes depends upon the temperature of the storage chamber, the relative humidity, and the air currents in the room.

In the chilling method the temperature is kept just above the freezing point. This is the condition encountered in the usual ice or electric refrigerator in the home. The physical state of the food is unaltered. Eggs, vegetables, and fruits are better chilled rather than frozen solid.

Cold storage is preferable to chilling from a bacteriological standpoint, because there is less bacterial action when the food is frozen solid. Chilling retards but does not prevent bacterial action. Chilled foods cannot be kept for many weeks.

Cold does not kill all species of bacteria, but it does slow down their metabolic activities. This means that the organisms multiply at a very slow rate. Few bacteria are capable of multiplying below 10°C. These low-temperature organisms are referred to as psychrophilic (cold-loving) forms.

Molds are able to multiply under conditions that prevent the growth of bacteria. Some species of molds are capable of growing on the surface of meat kept at temperatures below the freezing point, provided sufficient moisture is present in the air. In the absence of sufficient humidity the molds lose moisture and die. For the prevention of mold growth it is necessary to keep the temperature and humidity low. The temperature and humidity necessary to prevent the growth of molds varies with different types of foods.

Molds do little damage to foods. The chief objection to their presence is that they produce considerable discoloration. Under some conditions their metabolic waste products may leave unpleasant tastes and flavors in the food but they are usually harmless.

Drying.—The preservation of foods by drying or dehydration is of ancient origin and, although it was not greatly employed at first, the process is now of great industrial importance. Foods that are now preserved in this manner include nuts, seeds of legumes, grains, raisin grapes, prunes, figs, dates, apples, apricots, peaches, cherries, cranberries, potatoes, carrots, eggs, milk, fish, meat, etc.

Dried foods resist attack by yeasts, molds, and bacteria, and the enzymes elaborated by them or present in the foods, because the amount of moisture is insufficient to support growth or enzymatic activity. Furthermore, the organisms themselves lose water by drying and are rendered incapable of activity. Since the organisms are not necessarily killed, they may remain in an inactive form for long periods of time. If the moisture content is increased, spoilage may occur in a short time. This is especially true of the molds, which are able to tolerate a much lower moisture content than either the yeasts or the bacteria.

Dehydration of foods is a valuable procedure for several important reasons. Dried foods may be easily preserved for future use. This means that certain foods may be utilized over longer periods of time rather than for only a short season of the year. Dehydration greatly reduces the bulk of a product, conserves space, and facilitates handling. This is a decided advantage from the standpoint of transportation costs. Most of the dehydrated products, if properly prepared, are very good substitutes for fresh foods, being detected from the normal product with difficulty. Dried foods do not require sterilization or the maintenance of sterile conditions during preparation. They are more economical to

use since no waste is involved. Only that amount necessary for use at one time need be prepared.

The use of dehydrated foods also presents several decided disadvantages. Dried products require a long soaking period to restore the water lost by evaporation. The period required for rehydration varies with different foods. If this is not carefully done, the results are likely to be unsatisfactory. Sometimes dehydrated foods become infested with insects due to improper packaging or handling. Sometimes dried foods become moistened, with the result that conditions become favorable for the growth of bacteria, yeasts, and molds. This applies more especially to the hygroscopic foods or those which readily absorb moisture from the air.

Preservatives.—Sometimes chemicals are added to foods to preserve them. These act by killing the organisms or merely preventing them from multiplying.

An ideal antiseptic would be one that killed microorganisms or prevented them from multiplying without producing any harmful physiological effect. Apparently such a compound is not yet known. All of the commonly used preservatives exert some physiological action on the human body and, unless employed in minute amounts, may produce harmful effects.

The inorganic chemicals commonly employed include boric acid and borax, nitric acid and nitrates, nitrous acid and nitrites, sulfurous acid and sulfites.

Boric acid is a weak antiseptic, saturated solutions being unable to destroy bacteria. However, it does prevent the growth of most bacteria. It is sometimes added to butter as a preservative.

Sodium nitrate (saltpeter) and small amounts of sodium nitrite are usually added to salt solutions used for the pickling of meats. The nitrate was believed to react with the hemoglobin of the meat to give nitrosohemoglobin. This compound imparts a bright red color to the meat, making it more attractive in appearance. It is true that the bright red color is nitrosohemoglobin but it is not the result of the reaction between nitrate and hemoglobin but rather between nitrite and hemoglobin. Some bacteria present on meat are able to reduce nitrate to nitrite. The same or other species produce small amounts of organic acids from the meat which convert the nitrate and nitrite to nitric and nitrous acids respectively. The nitrous acid then reacts with the hemoglobin to give nitrosohemoglobin. An acid solution is necessary for the reaction to take place. The nitrate and nitrite also produce an inhibitory effect on the growth of bacteria likely to be present on meat. The nitrite has been shown to be more effective in this respect than the nitrate.

Hall (1935) recommended a new method for pickling meats. He used a pickling solution containing salt, nitrate, nitrite, and a small amount of citric acid or some other appropriate acid. The purpose of the acid is to convert the nitrate and nitrite into nitric and nitrous acids. The nitrous acid is then capable of reacting with hemoglobin to produce the attractive red color.

Sulfurous acid and sulfites are added to alcoholic liquids, especially wines. The addition of sulfite imparts an attractive red color to meat. It enjoyed great popularity as an addition to hamburger prepared from old, scrap meat. The beautiful red color restored the fresh appearance to the meat. Its use for this purpose is now prohibited by law.

The organic chemicals added to foods include benzoic acid and benzoates, salicylic acid and salicylates, formaldehyde, and creosote. Benzoic acid and benzoates are used for the preservation of vegetables. A small amount of sodium benzoate is sometimes added to tomato catsup to preserve the contents after the bottle is once opened. Salicylic acid and salicylates are used as preservatives of fruits and vegetables. Formaldehyde was formerly used as a preservative of milk and cream but its use for this purpose is forbidden by law. The use of wood smoke in the curing of meats is due to the presence of small amounts of creosote furnished by the burning wood.

High Osmotic Pressures.—Some foods resist the action of organisms in the presence of appreciable amounts of moisture. This applies to those foods containing high concentrations of sugar or salt. These compounds act by osmosis or the withdrawal of water from the protoplasm of the organisms. This causes a shrinkage of the protoplasm, which results in the death of the cell.

Practically no multiplication occurs in salt concentrations of 25 per cent or greater. Bacteria that are capable of resisting high osmotic pressures are called halophilic (salt-loving) organisms. A strength of 10 per cent markedly inhibits the growth of the great majority of bacterial species. The pathogenic or disease-producing organisms are less resistant to strong saline solutions than the saprophytic bacteria. Cane sugar in a concentration of 60 to 70 per cent usually prevents the growth of all types of microorganisms. Occasionally molds may be seen growing on the surface of a closed jar of jam or jelly. This is due to the fact that water evaporates from the jam or jelly and, not being able to escape, condenses back to water, producing a layer of less concentrated sugar solution on the surface. Some molds are capable of multiplying in this less concentrated solution.

Bacteria are not so sensitive to osmotic changes as are higher plant and animal cells. For this reason solutions having extremely high osmotic pressures must be used to kill bacteria.

It can be seen that the preservation of foods by the addition of sugar or salt produces the same effect as the removal of water. In both cases the ability of the food to resist attack is due to an increase in the osmotic pressure above the range compatible to the growth of the microorganisms.

BACTERIOLOGY OF MEAT

Contrary to earlier studies, newer results indicate that meat obtained from healthy, freshly slaughtered animals is seldom sterile. The organisms that have been isolated grow very slowly, visible signs appearing in from 7 to 14 days. Tissue removed under an anesthetic show about the same percentage of positive cultures as tissue obtained from animals slaughtered in the usual manner.

Organisms have been frequently isolated from the internal organs of freshly slaughtered animals. In most cases the numbers are exceedingly small, enrichment methods being required for their detection. Most of the organisms are probably the result of invasion of the tissues after the death of the animal.

Frozen or cold-storage meats can be kept for long periods of time without showing any signs of spoilage. On the other hand, meats kept at higher temperatures (chilled) show spoilage in much shorter periods. The changes that take place are the result of the action of autolytic enzymes normally present in the meat and those elaborated by the contaminating organisms. Proteins are first decomposed to amino acids and then putrefied with the liberation of bad odors. Fats and oils are hydrolyzed to fatty acids and glycerol. A short action of the proteolytic enzymes is beneficial in tenderizing meats but a prolonged action will result in decomposition and putrefaction.

The bacteria and molds usually found on meat are, with rare exceptions, not dangerous to health. The putrefactive changes produced by bacteria are objectionable from the standpoint of odor and taste and the growth of molds lowers the appearance of meat.

Weinzirl and Newton (1914*a*,*b*) examined samples of meats purchased in the open market and found that the numbers of aerobic organisms present varied from 270,000 to 88,000,000 per gram. This applied to ground meat (hamburger) as well as to tissue in larger pieces. They failed to judge the quality of meat by the number of organisms present. Others have reached a similar conclusion. There appears to be no correlation between the bacterial population and the sanitary quality of meat. This is due to the fact that it is not so much the numbers as it is the kinds of organisms that determine the sanitary quality of meat. Some samples showing high counts of saprophytic organisms may produce no harmful effects when ingested. Other samples having low bacterial

counts may produce harmful effects. Weinzirl and Newton suggested a standard limiting the number of organisms to 10,000,000 per gram of meat. They found that such a standard would condemn about 50 per cent of the hamburger sold in meat markets. Apparently the best criteria for judging the quality of meat is appearance, feel, and smell. This is sometimes referred to as the organoleptic test.

Both aerobic and anaerobic organisms are concerned in the spoilage of meats, fish, and other high-protein-containing foods. The aerobic

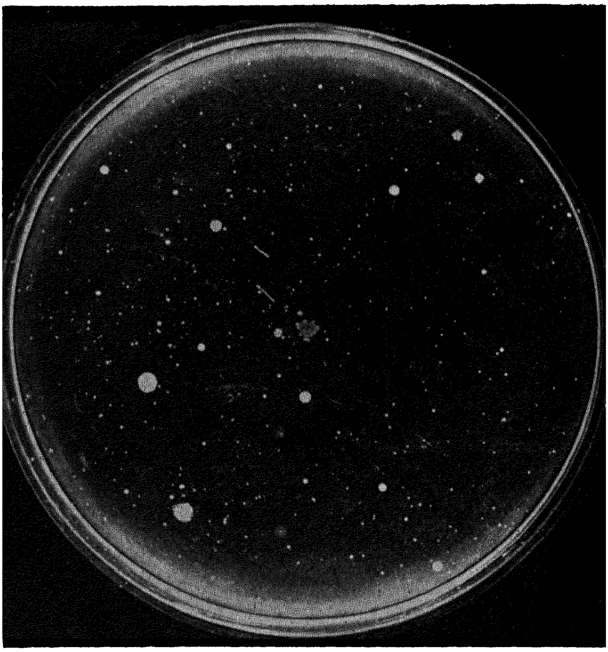


FIG. 166.—Ground meat. Organisms from 1 cc. of a 1:1000 dilution.

organisms act first and create an environment favorable to the growth of the anaerobes. Then the anaerobes attack the proteins and decomposition products released by the aerobes, resulting in the liberation of foul-smelling compounds. The process of putrefaction involves the action of both aerobes and anaerobes but the changes that occur are chiefly anaerobic in character.

Many types of organisms are concerned in putrefaction. These may be grouped as follows: (1) Gram-positive, aerobic, spore-bearing rods, (2) Gram-negative, aerobic, nonspore-forming rods, (3) cocci, (4) anaerobes, and (5) molds and yeasts. The Gram-positive, aerobic, spore-bearing group includes *Bacillus subtilis*, *B. albolactis*, *B. mesentericus*, etc. These organisms are saprophytes and are capable of liquefying

gelatin very rapidly. The Gram-negative, aerobic, nonspore-forming group includes *Escherichia coli*, *E. coli* var., *communior*, *Proteus vulgaris*, *P. mirabilis*, *Aerobacter cloacae*, and *Pseudomonas fluorescens*. The members of the genus *Proteus* are capable of initiating some of the changes produced by anaerobes. They are very proteolytic in their activities, being able to carry protein degradation farther than any of the other aerobes in this group. Some of the cocci that have been isolated include *Staphylococcus aureus*, *Micrococcus candidus*, *M. aurantiacus*, *M. candicans*, *M. saccatus*, *M. flavescens*, *M. roseus*, and *Sarcina aurantiaca*. All these organisms are Gram-positive. The most pronounced changes on meats are produced by the anaerobic, spore-bearing organisms. Some of the anaerobes that have been isolated from fresh and spoiled meat are *Clostridium perfringens*, *C. tertium*, *C. bifermentans*, and *C. sporogenes*. These organisms are responsible for putrefactive changes on proteins, resulting in the liberation of foul-smelling compounds. The molds that have been found growing on meat belong to the following genera: *Aspergillus*, *Penicillium*, *Mucor*, *Cladosporium*, *Sporotrichium*, *Alternaria*, and *Monilia*. Those belonging to the genera *Penicillium* and *Mucor* have been isolated with greater frequency than any of the others. Spores of such molds are commonly present in air. Several species of yeasts have been isolated from meat kept under refrigeration.

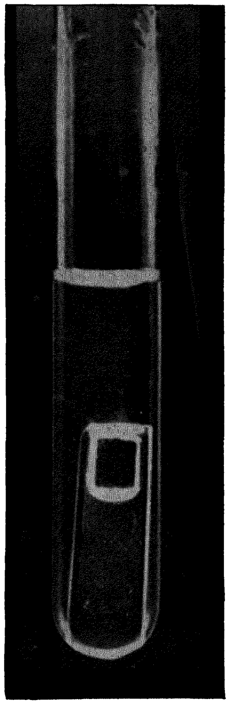


FIG. 167.—Ground meat. Acid and gas production in glucose fermentation broth.

Molds are aerobic and grow on or near the surface of meat. Most molds produce pigments that impart discolorations to meat. Molds may be removed by wiping or trimming the surface layer with a knife. If molds are permitted to grow without being checked, they may impart unpleasant odors and flavors to meat.

Dried Beef.—Meat is dried in order that it may be preserved for long periods of time. Dried beef is prepared in the following manner: Beef sets are obtained from the carcasses of freshly slaughtered animals and kept under refrigeration until ready to be used. The meat is usually cured in barrels or in tierces. In barrel curing about 4½ gal. of pickle is used for each 100 lb. of meat. In tierce curing about 300 lb. of meat and 8 to 12 gal. of pickle are added to each container. The length of the pickling period varies, depending upon the temperature. Sets are usually cured in from 75 to 120 days.

Beef ham sets must be well cured, otherwise decomposition may occur during the drying and smoking operation. Beef hams increase in weight about 8 per cent in the curing process. The pickle used is often plain brine and saltpeter, but frequently sugar and a small amount of sodium nitrite are added. A typical pickling solution has the following composition:

	Parts
Sodium chloride (NaCl)	25
Sodium nitrate ($NaNO_3$)	0.05
Sodium nitrite ($NaNO_2$)	0.10
Sugar (sucrose)	4
Water, to make	100

After the meat is cured it is soaked well to remove an excessive amount of salt. The soaking water is usually maintained at a temperature of 60 to 80°F. (16 to 27°C.). Two changes of water are usually employed. The length of the soaking period depends upon the kind of meat, nature of the pickle, length of time in the pickle, etc. Overcured, very salty, and hard-cured meats are soaked longer than mild-cured meats. The soaking period is usually from 12 to 24 hr. Meats are soaked for shorter periods in summer than in winter.

The meat is now ready to be dried, or dried and smoked. The meat is handled in a dry room heated by means of steam coils or by a hot-air furnace, and provided with good air circulation to remove moist air. By this method the drying may be completed in from 5 to 9 days. The room is kept at a temperature of 135°F. for the first two days, then dropped to 115°F. for the remainder of the drying period. Sometimes both drying and smoking are practiced. Wood smoke is produced and distributed in smokehouses in conjunction with heat and air circulation to preserve, color, and flavor cured meats. Smoke is produced by burning wood or sawdust. Hardwoods, such as hickory and maple, are used extensively and impart very desirable flavors to cured meats. Preservation is due not only to desiccation but to absorption of gases and fumes of creosote, pyroligneous acid (a mixture of acetic acid, acetone, methyl alcohol, etc.), and other antiseptic substances found in wood smoke. Thorough drying of the product is essential.

The final operation consists in chilling the smoked beef at a temperature of 34°F. (1°C.) so that it can be easily sliced. The outside slices show less moisture than those from the center. The sliced product is now ready to be packed for commerce.

SWEETENED CONDENSED MILK

Sweetened condensed milk is prepared in the following manner: Clean sweet milk is pasteurized at a temperature of 80 to 90°C. for about 1 min.

in order to kill most of the bacteria and inactivate the enzymes present in milk, which may cause undesirable physical and chemical changes. Cane sugar is added as dry crystals or in the form of a boiled, concentrated solution. The preparation is heated under a reduced pressure so that it will boil at a temperature of 50 to 60°C. The milk is reduced to almost one-third of its original volume. The final product contains about 25 per cent water, 40 per cent cane sugar, at least 8 per cent milk fat, and 28 per cent total milk solids including fat. The evaporated and cooled milk is transferred to sterile containers and capped.

Sweetened condensed milk is not sterilized before being placed in cans. The increased osmotic pressure of the preparation, due to the added sugar, is sufficient to prevent multiplication of most organisms. For this reason only a small percentage of cans of sweetened milk are ever sterile. Occasionally some cans show evidence of attack by organisms. The ends of the cans become blown, owing to the fact that the organisms produce gas from the added sucrose and from the lactose normally present in the milk.

The organisms that have been isolated include yeasts, molds, staphylococci, streptococci, diplococci, anaerobes, gas-producing rods, aerobic spore-forming bacilli, thermophilic and other aerobic bacteria. The coccus forms are encountered in practically every sample of sweetened condensed milk. These organisms are able to survive the temperatures employed in its preparation. Anaerobic organisms cause little trouble, being rarely found in condensed milk. Organisms of the *Escherichia-Aerobacter* group are occasionally encountered but offer little trouble. The medium is unfavorable and the rods ultimately die. There is no evidence that the aerobic gas-producing organisms are responsible for the blown appearance of cans showing fermentation. Bacilli of the aerobic, spore-bearing types are almost always found in both normal and decomposed cans of milk. These organisms are actively proteolytic and may initiate changes on the milk proteins. It is questionable that aerobic, sporulating rods ever produce swells in cans of sweetened, condensed milk. The cans contain an insufficient amount of oxygen and too much sugar for active growth. Organisms of this group play an insignificant role in the peptonization of sweetened, condensed milk. Thermophilic bacteria are often present. They are neither actively proteolytic nor gas producers and, therefore, play no part in the peptonization and fermentation of sweetened, condensed milk.

Yeasts are the most common organisms found in cans of condensed milk. Many of these organisms are active fermenters, attacking the sucrose with the liberation of gas. This results in the cans having a blown appearance. Not all species found in milk are capable of attacking sucrose. Unless the sugar is fermented, the presence of yeasts does not

mean that abnormal changes have taken place. Even the fermentative types grow with difficulty owing to the fact that the environmental conditions are not favorable. The sources of yeasts in canned milk are (1) contamination of the original fresh milk, (2) contamination of the air of the cannery, and (3) contamination of the added sugar. It is not believed that any of the yeasts found in milk are harmful to man. Decomposed milk is objectionable but practically free from any toxic substances.

BACTERIOLOGY OF EGGS

Fresh eggs are not always free from microorganisms. Results have shown that about 10 per cent of fresh eggs contain living viable bacteria. The yolks are infected more than the whites although in some eggs organisms have been isolated from both components. The organisms that have been isolated from contaminated eggs include various species of cocci, coccoid forms, rods, and molds. Fresh eggs appear to be free from such fermentative and proteolytic bacteria as *Escherichia coli*, *Proteus vulgaris*, and anaerobes. The bacteriology and mycology of eggs have not been studied sufficiently and systematically to give an accurate picture of the flora of contaminated eggs.

An egg shell is about $\frac{1}{60}$ in. in thickness and composed largely of calcium carbonate. It is a porous structure, the pores being sufficiently large to permit the passage of gases and microscopic solid particles. When fertile eggs are incubated, these pores are the means by which air passes through the shell to furnish oxygen to the developing chick embryo. Organisms are always found on the shells of eggs. The number varies from 400 to 1,600,000 per gram of shell. *Escherichia coli* is present on practically every shell. Damp and soiled eggs soon become contaminated owing to the fact that moisture aids in carrying organisms through the pores. This applies to molds as well as to bacteria.

Preservation of Eggs.—Several methods are employed for the preservation of eggs: (1) cold storage, (2) freezing, (3) drying, (4) immersion in sodium silicate solution (water glass), (5) packing in brine or sawdust, (6) coating with vaseline, (7) wrapping in oiled paper, (8) coating with paraffine, (9) immersion in lime water (solution of calcium hydroxide), etc.

In the cold-storage process the eggs are kept at a temperature of about $-6°C$. If the temperature goes below this point a nonreversible change takes place in the albumin, preventing the egg from being restored to its normal transparent condition.

Frozen eggs are used in large quantities by candy makers, bakers, egg noodle and macaroni manufacturers, and in other industries. The eggs are removed from the shell, placed in large metal containers, and

frozen. Often the whites and yolks are separated because they may be used for different purposes. Frozen eggs are kept at a temperature of about $-18°C.$, or lower, until needed. They are usually cheaper than fresh eggs because they may be prepared during the months of high production.

Eggs may be dried unseparated or first separated into whites and yolks and then dried. Dried yolks, whites, and unseparated eggs are used to a considerable extent in prepared cake and doughnut flours, in ice creams, in macaroni and noodles, in bakery products such as meringue and marshmallows.

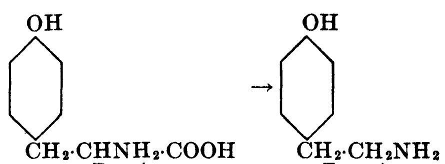
Dried eggs have good keeping qualities if kept dry and cool. However, the presence of a small amount of moisture and a warm atmosphere may be sufficient to permit the growth of organisms and cause spoilage in a short time. It is desirable, therefore, to keep eggs stored in a cool place and protected from an excessive amount of moisture.

The other methods used for preserving eggs are designed to prevent the passage of air (oxygen) through the pores of the shell and into the egg. Regardless of which one of these procedures is followed, the eggs must be kept under cold conditions to prevent decomposition by the enzymes normally present in the egg.

FOOD POISONING

Food poisoning in the light of newer knowledge refers to the ingestion of food contaminated either with harmful bacterial organisms or with certain soluble excretory products known as toxins. It does not include the toxic effect following the consumption of noxious plants, such as mushrooms or poisonous mussels, or of decomposed foods containing chemical poisons, or of idiosyncrasies connected with certain plant and animal poisons.

Previous to this, food poisoning was believed to be due to the consumption of food containing certain chemical compounds known as ptomaines. The term ptomaine is from the Greek and means a dead body. Ptomaines are produced in putrefying meat and in compounds of a similar nature. They are basic substances and belong to the group of compounds known as amines. They result chiefly from the decarboxylation of amino acids. A typical reaction is the following:



Tyrosine → Tyramine

The amino acid tyrosine loses carbon dioxide and is converted into the ptomaine tyramine. Ptomaines appear only when putrefaction is in an advanced stage. Ptomaines are poisonous when injected into the tissues but there appears to be very little evidence that they produce any toxic action when taken by mouth. The ptomaine theory of intoxication is a misconception.

The fact that bacteria are present in foods does not mean necessarily that they are harmful. Many saprophytic organisms can attack proteins and release ptomaines during the later stages of decomposition. However, most bacteria that are capable of putrefying proteins with the formation of ptomaines are harmless when taken by mouth. Putrefactive aerobic and anaerobic species normally present in the intestinal tract of man and capable of producing the above changes are harmless when ingested with food.

The organisms responsible for true food poisoning belong to three well-defined types: (1) members of the genus *Staphylococcus* (*S. aureus*, *S. albus*, etc.), (2) several types of *Clostridium botulinum*, chiefly type *A* in this country and type *C* in Europe, and (3) members of the genus *Salmonella* (*S. enteritidis*, *S. schottmuelleri*, *S. aertrycke*, etc.). *C. botulinum* and some strains of staphylococci produce their toxic effect by the secretion of soluble toxins. This is especially true of the former. These organisms probably never multiply within the body when ingested with foods. The diseases are essentially intoxications produced by the secretion of soluble exotoxins. The spores of *C. botulinum* are found in the soil and are likely to be present on many kinds of foods. If they are not destroyed in the heating process, conditions are usually created that permit germination of the spores. The vegetative cells then secrete a powerful toxin in the food. The mere tasting of such food may be sufficient to cause death. The toxin can pass through the walls of the stomach and intestine unchanged, differing in this respect from practically all bacterial toxins. Some strains of staphylococci are also able to grow in food and elaborate a soluble toxin. The ingestion of such food produces an intoxication but the symptoms are considerably less severe than those produced by *C. botulinum*. Death rarely follows ingestion of food containing staphylococcus toxin. Members of the genus *Salmonella* produce chiefly endotoxins. The organisms are ingested with the food and reach the intestinal tract without being destroyed. They multiply in the intestines and liberate their endotoxins following death and digestion of the bacterial cells.

Spoiled canned foods or those which are suspicious of spoilage should never be tasted. If the container is contaminated with *C. botulinum*, sufficient toxin may be present in a minute portion of the food to cause death. The suspected food should not be fed to animals or chickens

TABLE 48

General food poisoning	Botulism
INCUBATION PERIOD	
Usually 2 to 4 hr., staphylococcus toxin. If over 12 hr., salmonella bacilli	Usually 24 to 48 hr.
TREATMENT	
Supportive and eliminative	Botulinus antitoxin, specific type; absolute quiet; eliminative
INVESTIGATION PROCEDURE	
1. Use incubation period for basis of determining the causative meal	1. Use incubation period for basis of determining the causative meal
2. Always suspect freshly cooked or warmed-over foods, cakes, pastry, minced meats, etc. Preserved foods are rarely at fault. The foods are apparently all right as to taste, appearance, odor, and texture	2. Always suspect preserved foods; likewise, meat products such as sausages. Spoilage of foods is noted in many instances
3. Bacteriologic examination of excreta of patients and the suspected food for the salmonella group, staphylococcus group, and other organisms	3. Test suspected food for toxin by animal inoculation; mice, guinea pigs, or rabbits. Test for type with specific antitoxin. Cultures of suspected foods for the presence of spores, particularly if food has been previously boiled
4. Bacteriologic and epidemiologic search for human carriers and possible contamination from animal sources	4. Search for domestic animals, such as chickens with symptoms of limberneck, for corroborative field and laboratory evidence
5. Complications: appendicitis, cholecystitis, persistent elevation of temperature (paratyphoid infection)	5. Complications: bronchopneumonia
	6. Human outbreaks are usually due to type *A* toxin
SYMPTOMATOLOGY	
Sudden onset; nausea, vomiting, abdominal pain, prostration, diarrhea, and rise of temperature	Delayed onset; marked muscular weakness; gastrointestinal symptoms rare; disturbances of vision with diplopia and blepharoptosis; loss of ability to swallow and talk; constipation; rapid pulse and subnormal temperature; rarely any pain; death from respiratory failure
Mortality, 0 to 1 per cent Case-infectivity rate high	Mortality over 60 per cent Case-infectivity rate usually 100 per cent

as they may be highly susceptible to the toxins, especially that secreted by *C. botulinum.* Spoiled canned foods are best disposed of by thorough cooking to destroy the toxin.

According to Meyer, and as reported by Cruess (1935), the incubation period, treatment, investigation procedure, and symptomatology of the three types of organisms responsible for food poisoning are given in Table 48.

For additional reading on foods consult Hoffstadt (1924*a*,*b*), Medical Research Council (1929), Tanner (1932), Canning Trade (1936), Bitting (1937), Prescott and Proctor (1937), Cruess (1938), and American Can Company (1939).

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CHAPTER XXIII

BACTERIOLOGY OF SOIL

Generally speaking, soils are excellent culture media for the growth of many kinds of organisms. This is especially true of the cultivated and improved soils. The microscopic life of the soil includes bacteria, yeasts, molds, algae, diatoms, and protozoa. The latter includes amoebas, flagellates, ciliates, and rotifers. In addition, there are present various nematodes, insects, etc.

Since most of the soil inhabitants are aerobic, the organisms are found in greater numbers in the surface layers. The numbers decrease as the depth of the soil increases. A well-aerated soil contains more organisms than one lacking in an abundance of oxygen. The numbers and kinds of organisms found in soil depend upon the nature of the soil, the depth, season of the year, state of cultivation, reaction, amount of organic matter, temperature, moisture, etc.

Methods are available for counting the numbers of organisms in soil as well as for isolating the various species in pure culture. Since the organisms may vary considerably in their growth requirements, many types of culture media must be employed. The organisms may be obligately aerobic, anaerobic, or facultative types.

Many of the species of organisms present in soil grow in association with others. The phenomena of symbiosis, synergism, and commensalism are believed to be of common occurrence. This explains why it is exceedingly difficult to determine from laboratory studies on pure cultures what actually takes place in the soil. Two species growing together frequently secrete metabolic waste products not produced by either organism growing in pure culture. Also, a product of metabolism of one organism may serve as a nutrient for another species (see Chap. XVIII for a discussion of the various types of bacterial associations).

The same general media and methods employed for the cultivation and isolation of the heterotrophic organisms are followed for the propagation and separation of the great majority of organisms found in the soil. However, the soil contains some species that are specific and do not grow on the usual laboratory media. Special media and methods must be employed for their cultivation. Specific soil organisms that have been isolated by the employment of special culture media include symbiotic nitrogen-fixing bacteria, nonsymbiotic nitrogen-fixing bacteria, sulfur-

oxidizing forms, sulfate-reducing species, urea decomposing forms, cellulose-decomposing organisms, hydrogen bacteria, methane bacteria, etc.

Under some conditions solid media are preferable to liquid preparations for the isolation of specific soil forms. An inorganic solidifying agent is usually more desirable than an organic gel. For this purpose silica gels are frequently used instead of agar. Such gels are prepared by treating sodium silicate or water glass with a known quantity of hydrochloric acid and then allowed to gel. The medium consists of a solution of inorganic salts in a silica gel base.

Functions of Soil Organisms.—One of the most important functions of soil organisms is to decompose various kinds of waste organic matter of plant and animal origin. This includes stable manures, green manures, plant stubble, plant roots, organic fertilizers, and other products. The decomposition of such compounds is the result of the activities of bacteria, molds, protozoa, worms, etc. Each group selects certain constituents of the organic matter suitable for building up its own characteristic protoplasm.

The organic compounds added to the soil as the result of biological action include various sugars, amino acids, pentosans or compounds that yield pentoses on hydrolysis, celluloses, lignins, proteins, fats, waxes, tannins, pigments, etc. These compounds are further decomposed, resulting in the liberation of soluble organic and inorganic constituents, which are made available by the action of the soil population. Some of the inorganic compounds, notably ammonia, may be utilized by plant life as a source of nitrogen.

Nitrogen Cycle.—A continuous transformation of nitrogen takes place in the soil by various groups of organisms. The first step in protein breakdown is a hydrolysis of the molecules to their constituent building stones or amino acids. The amino acids are then deaminized with the liberation of ammonia. The reaction is as follows:

$$\begin{array}{c} CH_3 \\ | \\ HCNH_2 \\ | \\ COOH \\ \text{Amino acid} \\ \text{(alanine)} \end{array} + H_2O \rightarrow \begin{array}{c} CH_3 \\ | \\ HCOH \\ | \\ COOH \\ \text{Hydroxy acid} \\ \text{(lactic acid)} \end{array} + \begin{array}{c} NH_3 \\ \text{Ammonia} \end{array}$$

A portion of the liberated nitrogen is utilized by the organisms to build up their own specific protoplasm. Some may be utilized by plants for the synthesis of proteins. The nonnitrogenous portion of the molecule is used largely for energy purposes.

After ammonia has been liberated from various nitrogenous compounds it may be (1) assimilated by soil organisms and again synthesized into proteins, (2) used by higher plant life as a source of nitrogen, (3)

absorbed by the colloidal substances in soil and bound as ammonia, (4) acted upon by other soil forms and oxidized first to nitrites and then to nitrates. The organisms responsible for this last set of reactions belong to the autotrophic group of bacteria. These organisms are incapable of utilizing organic compounds for structural and energy purposes.

The nitrates may be utilized by various microorganisms and by higher plants and synthesized into proteins, or they may be reduced first to nitrites and finally to free nitrogen. The free nitrogen is lost as far as

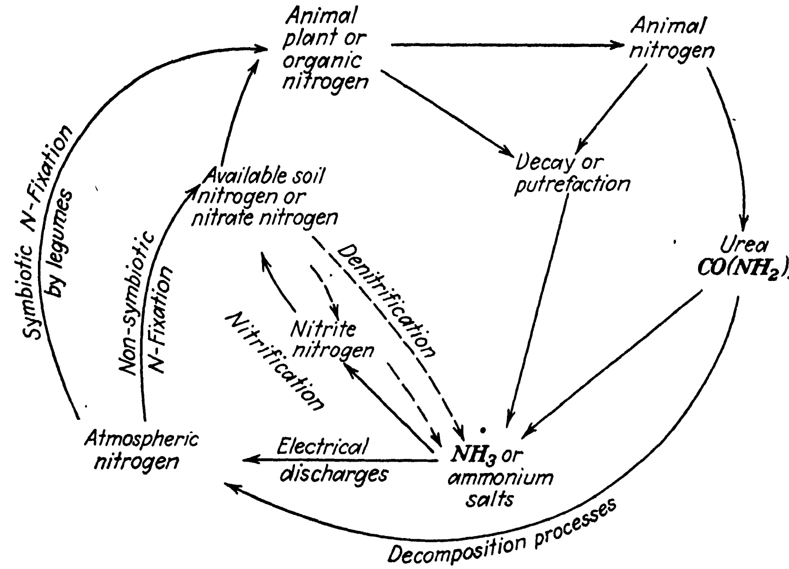


FIG. 168.—The nitrogen cycle. (*After Allen.*)

being available to plant and most microscopic soil life. However, certain bacteria found in the soil have the ability of utilizing free atmospheric nitrogen and making it available to plant life.

Two groups of organisms are responsible for nitrogen fixation. The organisms in one group are the nonsymbiotic nitrogen fixers; those in the other group fix nitrogen only when growing in symbiosis on the roots of certain plants. The free nitrogen is transformed into nitrogenous compounds and made available to plants and soil microorganisms. The nitrogen is again synthesized into proteins. The nitrogen cycle, according to Allen (1935), is diagramed in Fig. 168.

QUANTITATIVE EXAMINATION OF SOIL

Soil is the natural habitat of a considerable number of bacterial species. The organisms vary from strict autotrophs to heterotrophic

forms. Because of this fact, many types of culture media are required in order to obtain an accurate count of the numbers of organisms present. Such a procedure, however, would be out of the question where a rapid determination is desired of the approximate numbers present in a sample of soil.

✓ **Soil Colloids.**—Bacterial organisms are found chiefly in the layer of colloidal material surrounding the inorganic particles of soil. Conn and Conn (1940), McCalla (1940), and others noted that bacteria grew better in the presence of colloidal clay than in its absence. McCalla attributed the stimulation of bacterial growth to the catalytic effect of the clay in speeding up biochemical reactions, either by providing a

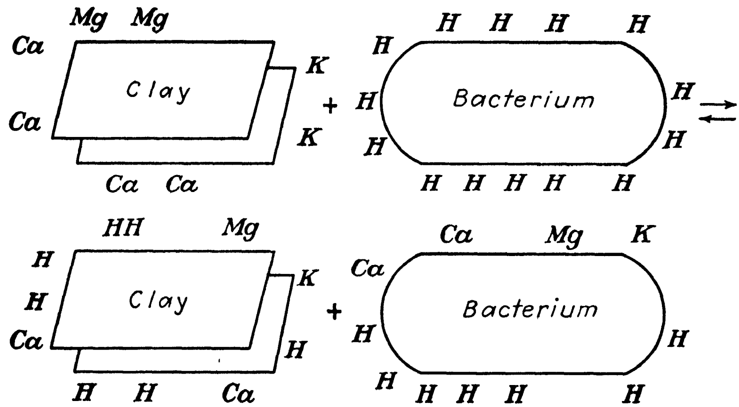


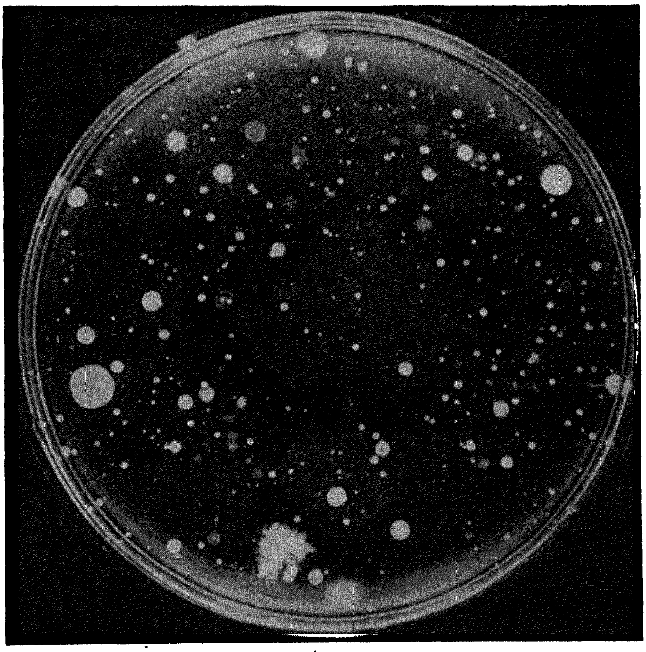
FIG. 169.—Mechanism of ion exchange between soil bacteria and colloidal clay. (*After McCalla.*)

more efficient utilization of nutritive material or by decreasing the toxic effects of waste products by adsorbing them.

Bacteria in contact with soil colloids adsorb cations. Under normal conditions the solid material in the soil constitutes about 80 per cent and the water content about 20 per cent. The water is present around and between the particles of soil. Normally the bacteria probably live in the water films that adhere to the surface of the colloid particles containing adsorbed ions. To quote from McCalla,

> The bacterial cell is undoubtedly in close proximity to the soil particles and assuming that the bacteria may adsorb ions and hold some of them in the outer surface of the cell, this would permit contact exchange of adsorbed ions. Ions with large oscillating volumes would overlap, and exchange between systems could readily take place. Other ions which are strongly adsorbed would not be expected to wander far from the surface of the colloid. On the basis of the displacement of adsorbed methylene blue the ions would be expected to be adsorbed by the bacteria from the soil colloids in the following series: H > Al > Fe > Mn > Ba > Ca > Mg > K > NH_4 > Na.

From the foregoing facts and theoretical considerations it is suggested that in the adsorption of nutrients from the soil by the bacteria, and possibly by living cells in general, an exchange of adsorbed bases takes place between the bacteria and soil colloid as depicted in Fig. 169. In bacterial metabolism large amounts of carbon dioxide and water are formed. In the presence of H_2O H-ions are produced from the carbon dioxide which may be adsorbed at the cell's surface. When a colloidal clay particle, saturated with adsorbed bases contacts a bacterium saturated with H-ions, an exchange of ions takes place until an equilibrium is reached. As this equilibrium is upset by the more complete utilization of the adsorbed basic ions in bacterial metabolism a further exchange may take place, the colloidal clay functioning as a constant reservoir for basic ions utilized in the growth of such organisms.

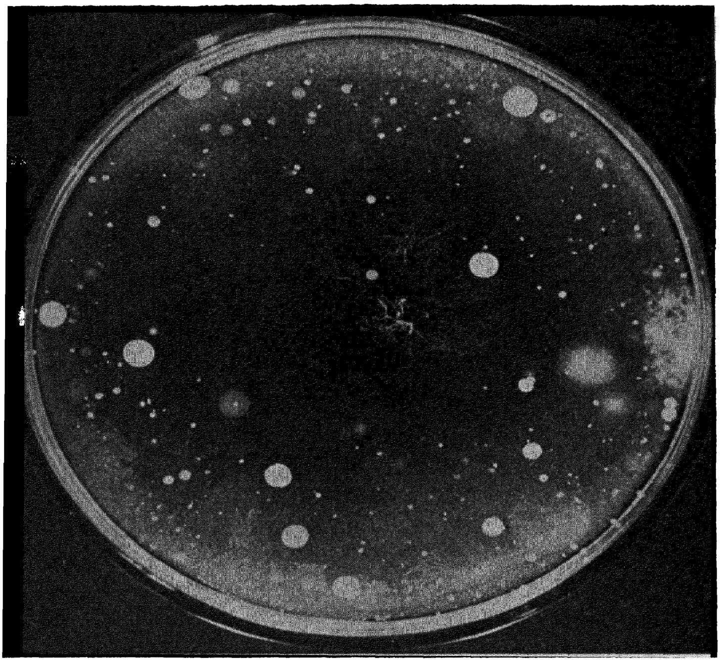


A

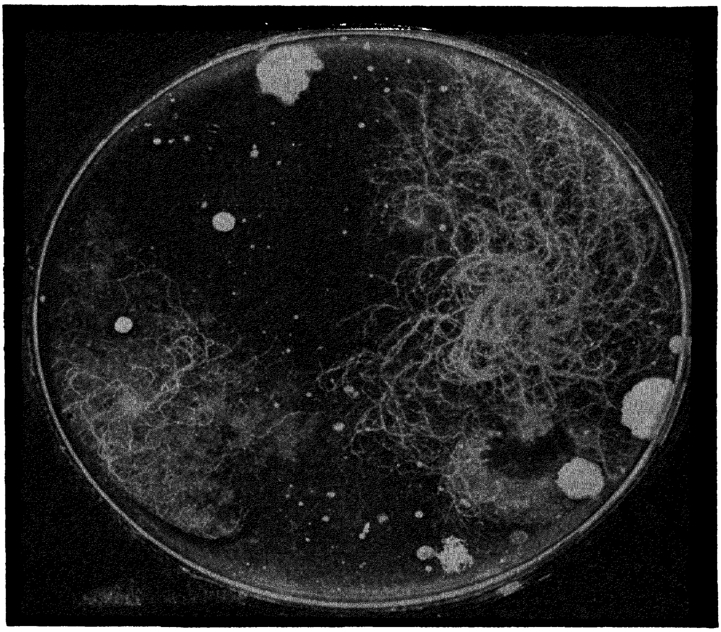
FIG. 170.—Quantitative examination of soil. *A*, count of top soil; *B* (p. 488), count of soil 1 ft. from the surface; *C*, count of soil 2 ft. from the surface.

It is difficult to remove or separate the organisms from this colloidal layer. This means that the number of colonies appearing on an agar plate is not an accurate index of the population of a soil sample. Also, no single culture medium is satisfactory for the growth of all species present in the soil.

Three methods may be followed to determine the numbers of microorganisms in the soil: (1) the plate method, (2) the dilution method, and (3) the direct microscopic method.



B



C

Fig. 170.—For descriptive legend **see p. 487.**

Plate Method.—The plate method has been followed more than any other procedure. A weighed sample of soil is mixed with a known volume of sterile water. The sample is well shaken to wash out as many organisms as possible from the colloidal material surrounding the soil particles. A series of dilutions are usually prepared from the first soil suspension. Aliquot portions from each dilution are transferred to Petri dishes and mixed with melted agar. The plates are incubated from 2 to 14 days. The colonies appearing on the plates are counted and computations made for the number of organisms per gram of soil (Fig. 170*A*,*B*,*C*).

The enumeration of the soil population by this method presents several very serious errors. Conditions are not favorable for the growth of the anaerobic soil organisms. The autotrophic bacteria do not multiply on an organic medium. The nonsymbiotic, nitrogen-fixing organisms grow to a limited extent only. Many of the cellulose-decomposing organisms fail to grow on the commonly used laboratory media. The counts as determined by this method represent only a fraction of the total bacterial population of the soil. It does, however, give an accurate index of the number of organisms present in the soil that are capable of growing on the commonly used organic culture media.

Dilution Method.—In this method a suspension of soil is prepared as already given. Aliquot portions of the suspension are transferred to different types of culture media favoring the growth of the specific soil forms. A urea medium is used for the growth of the urea-decomposing organisms; a cellulose medium is employed for the detection of the cellulose-hydrolyzing bacteria; a medium devoid of nitrogen is used for the isolation of the nitrogen-fixing organisms; etc. The accuracy of the method depends upon the preparation of a series of dilutions of the original soil suspension. Thus, if a dilution of 1:10,000 failed to show the presence of urea-decomposing bacteria whereas a dilution of 1:1000 did, then the number of organisms capable of liberating ammonia from urea would be between 1000 and 10,000 per gram of soil. More accurate counts could be obtained by employing a larger number of dilutions over a narrower range. This method is to be preferred for the isolation of the specific forms present in the soil.

The numbers of organisms found in soil by the agar plate method vary from 200,000 to 100,000,000 per gram. The counts are considerably less than the actual numbers present, owing to the limitations already mentioned. According to Waksman (1932), the colonies appearing on agar plates consist of 10 to 40 per cent actinomyces, 50 to 80 per cent nonspore formers, and 3 to 10 per cent spore-forming bacteria.

Molds are commonly present in soil although the numbers appearing on agar plates show only a small percentage of the total counts. Yeasts

are even less prevalent than molds, their numbers increasing in acid soils and in vineyards and orchards.

✓ **Direct Microscopic Method.**—The direct microscopic procedure is considered more accurate than either of the cultural methods already mentioned for the determination of the abundance of the soil population.

The method is as follows: One part of soil is suspended in 10 times its weight of a 0.015 per cent solution of gelatin in water. Agar may be used in place of the gelatin. The purpose of the agar or gelatin is to fix the organisms to the slide. The concentration of gelatin must not be above 0.015 per cent, otherwise the whole background of the smear assumes a pink color when stained. This does not apply to the agar, however, which may be employed in higher concentrations. A known volume of the soil suspension is placed on a ruled glass slide and spread uniformly over the marked area. The slide is dried on a flat surface over a water bath and then covered with a staining solution consisting of 1 per cent rose bengal dissolved in a 5 per cent aqueous solution of phenol. The slide is finally washed in tap water, dried, and examined under the oil-immersion objective.

In a good preparation the bacteria take a deep pink or red color whereas the mineral constituents do not stain. Some of the dead organic matter appears light pink, but most of it stains either yellow or not at all. If the bacteria appear faintly stained or if everything is colored pink, a new preparation should be made. The former condition generally means that the slide has been washed too long, whereas the latter indicates that the concentration of gelatin in the fixative is too great or the staining solution too old.

The direct counts are from 5 to 20 times greater than by the agar plate method. Much of the discrepancy is due to bacteria that fail to grow on the plates rather than to the presence of large clumps of organisms that do not break up in the plating process. Also, the direct method records dead organisms that are eliminated in the agar plate procedure.

The direct microscopic method for the enumeration of soil organisms is subject to several serious errors, especially in the hands of the inexperienced worker. The organisms must be evenly distributed over the slide. It is of great importance to prepare smears as uniformly as possible. It is usually considered advisable to prepare several slides and take an average of the counts. Another disturbing factor is the great difficulty in recognizing the bacteria. Many of the soil forms are too small to be easily distinguished from the soil particles. Also, many particles of soil resemble bacteria. Considerable experience is, therefore, necessary in making accurate determinations of the soil population by the direct method.

For more information consult the reports by Conn (1918, 1926).

Variations in Soil Counts.—The numbers of organisms in a soil are not uniform even over a very small area. In order to increase the accuracy of the determination several samples from the same plot of ground should be collected and an average taken of all the soil determinations for computing the final soil count. A single determination may be considered as valueless for computing the soil population.

Soil counts have been shown to vary from day to day. These daily changes appear to be related to fluctuations in the numbers of active protozoa. Russell and Hutchinson (1909, 1913) found that, if soil were partly sterilized with steam or with a volatile antiseptic such as toluene, the bacterial counts showed first a decrease followed by a sharp increase in numbers and activity. If a little untreated soil were added, the bacterial counts again decreased. The partial sterilization of the soil destroyed the protozoa but not the bacteria, and was followed by a sharp increase in the latter. The addition of an unheated soil reintroduced protozoa and resulted again in a decrease in the numbers of bacteria. The important protozoa responsible for the daily variations in the bacterial counts include ciliates, flagellates, and rhizopods. These organisms have been found to be of world-wide distribution in the soil.

Cutler, Crump, and Sandon (1922) found that an inverse relationship existed from day to day between the numbers of bacteria and active amoebas in the soil. A decrease in the numbers of protozoa was followed by an increase in the bacterial population, and vice versa. This means that, if a soil analysis is to be of any value, it is of great importance to examine samples from different parts of the same plot as well as at frequent intervals over a long period of time.

QUALITATIVE EXAMINATION OF SOIL

Lochhead (1940) reported that the qualitative nature of the soil microflora was markedly influenced by the growing plant. In the rhizosphere (the zone influenced by root excretions) the Gram-negative rods were increased in numbers and the Gram-positive rods, coccus forms, and spore bearers were less abundant.

Organisms in the rhizosphere showed greater physiological activity than those more distant from the plant. There were present, (1) a greater proportion of motile forms, (2) a pronounced increase in the number of chromogenic bacteria, and (3) a higher incidence of organisms capable of liquefying gelatin and of attacking glucose.

In a later report West and Lochhead (1940) stated that bacteria of the rhizosphere of flax and tobacco showed more complex nutritive requirements than those of the control soils. The roots were found to stimulate the growth of those types which required thiamin (vitamin B_1), biotin (vitamin H, bios II B, coenzyme *R* factor), and amino nitrogen for

growth. This suggested that the plant roots excreted significant amounts of these essential substances. The difference in the microflora of the rhizosphere and control soil was more pronounced where the latter was poor than where it was supplied with an abundance of organic matter. This was due, no doubt, to the liberation of growth substances in the decomposition of the organic matter, which were essential for the growth of the typically rhizosphere forms.

AMMONIA PRODUCTION

Most species of bacteria are capable of decomposing proteins and protein split products with the liberation of ammonia as one of the compounds. The ammonia is released prior to the utilization of the organic acid residue chiefly for energy purposes. The production of ammonia is an essential stage in the formation of nitrate in the soil. Most plant crops are dependent largely upon soil nitrates for structure and growth.

It is important to note that the decomposition of nitrogenous compounds for energy takes place only in the absence of an easily utilizable carbohydrate. In the presence of a readily available carbohydrate, bacteria derive their energy from this source, utilizing the nitrogenous compounds for structure and growth only. This results in a greatly lowered production of ammonia and nitrate. Therefore maximum ammonia production takes place only in the absence of a readily available carbohydrate.

Proteins and their degradation products are not the only compounds capable of liberating ammonia following bacterial attack. Some nitrogenous compounds of a nonprotein nature are also acted upon by bacteria, resulting in the release of free ammonia.

Stable and barnyard manures are often used as fertilizers because of their nitrogen content. They help to replenish the nitrogen supply of the soil. A high content of urine is often present in such waste material. The most important nitrogen compound present in urine is urea. Many organisms have the power of converting the urea to ammonium carbonate and finally to free ammonia and carbon dioxide, according to the equation,

$$O{=}C\begin{matrix} \diagup NH_2 \\ \diagdown NH_2 \end{matrix} + 2H_2O \rightarrow (NH_4)_2CO_3$$

$$(NH_4)_2CO_3 \rightarrow 2NH_3 + CO_2 + H_2O$$

The presence of urea decomposing organisms in the soil is determined by incorporating urea in the culture medium. The liberation of ammonia from the culture medium indicates the presence of the specific organism

or organisms capable of utilizing this nitrogenous compound. Many bacterial species are capable of converting urea to ammonium carbonate. Among these may be mentioned: (1) *Micrococcus ureae*, a Gram-positive, aerobic coccus appearing singly, in pairs and in clumps. Its optimum temperature is 25°C.: (2) *Bacillus freudenreichii*, a Gram-positive, motile, facultative aerobic, spore-forming rod. The cells appear singly and in chains. The optimum temperature is 30°C.: (3) *Sarcina ureae*, a Gram-positive, motile, aerobic coccus occurring singly, in pairs and in packets. The organism does not produce spores but resists heating to 80°C. for 10 min. The optimum temperature is 20°C.: (4) *Pseudomonas ureae*, a Gram-positive, motile, facultative aerobic rod. The cells occur singly and in pairs. Its optimum temperature is 20°C.

The urea bacteria usually thrive best in media containing urea, especially when made alkaline with ammonium carbonate. The organisms are capable of rapidly converting the urea to ammonium carbonate, resulting, in many instances, in the death of the bacteria. The organisms are commonly found in air, water, soil, and manure. About 2 per cent of the organisms present in surface soil and about 10 per cent of those present in manure are capable of decomposing urea. The isolation of the urea-decomposing organisms is a simple matter, provided urea is added to the culture medium.

DIGESTION OF CELLULOSE

The greater part of the organic matter in the soil is decomposed by bacteria in the process of [illegible] energy. The simpler carbohydrates, including the monosaccharides, the disaccharides, and some of the polysaccharides are easily attacked and decomposed by a large number of soil bacteria. The addition of such compounds to the soil causes a rapid increase in the bacterial population.

The digestion of cellulose has attracted considerable attention, owing chiefly to the fact that this compound is one of the most important constituents added to the soil. Cellulose is a polysaccharide and yields glucose on hydrolysis. The cell walls of plants are composed of true cellulose whereas those of animals are made up of protein. This is a distinguishing characteristic between plant and animal cells.

Organisms Concerned.—Cellulose is attacked by both aerobic and anaerobic bacteria. The aerobic organisms appear to be of greater importance than the anaerobic in the decomposition of cellulose. The presence of aerobic cellulose-digesting organisms can be easily demonstrated by placing some soil in a culture medium composed of inorganic salts and cellulose (strip of filter paper) and incubating at room temperature for several days. The filter paper slowly disintegrates and finally disappears completely (Fig. 171).

The first stage in the utilization of cellulose is a hydrolysis of the molecule to cellobiose and finally to glucose. The two enzymes cellulase and cellobiase are concerned in the reaction. Cellulase hydrolyzes cellulose to cellobiose and cellobiase splits cellobiose to two molecules of glucose (see pages 243 and 246).

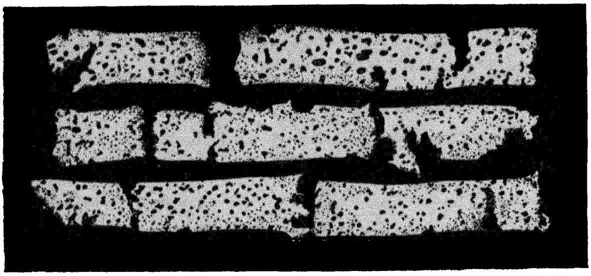


FIG. 171.—Strips of filter paper showing disintegrated areas due to bacterial attack. (*After Omelianski; from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

The two enzymes cellulase and cellobiase accompany each other. The enzymes are of tremendous importance in the dissolution of insoluble cellulose in the soil. Since animals do not elaborate a cellulase, the presence of cellulose-decomposing bacteria in the intestines of herbivorous animals is responsible for the hydrolysis of the insoluble compound, making it available as food.

Omeliansky (1904) showed that cellulose may be hydrolyzed by two spore-bearing, anaerobic organisms, which he designated the hydrogen bacillus and the methane bacillus (Fig. 172). He separated the two organisms from a mixed culture and found that one produced only the hydrogen fermentation whereas the other produced only the methane fermentation.

FIG. 172.—Cellulose-digesting organisms. 1, 2, 3, hydrogen bacillus; 4, 5, 6, methane bacillus. (*From Lipman, "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

Hutchinson and Clayton (1919) isolated a pleomorphic organism, which existed as a coccus, as a motile filamentous form, and as an oval spore-like body. They named the organism *Spirochaeta cytophaga* (*Cytophaga hutchinsonii*). The organism grows poorly or not at all in the presence of organic matter but grows well in a medium composed of inorganic salts to which is added cellulose as a source of carbon.

Khouvine (1923) reported the presence of an obligately anaerobic, spore-forming bacillus in the human intestine, which attacked cellulose

with the formation of acetic acid, accompanied by smaller amounts of butyric acid, ethyl alcohol, carbon dioxide, and hydrogen. Cellulose served as the only source of carbon. The organism could be cultivated in an inorganic salt medium containing 0.1 per cent peptone, about 1 per cent cellulose, and some fecal extract. The organism failed to develop on a medium in which cellobiose or glucose was substituted for the cellulose. He named the organism *Bacillus cellulosae dissolvens* (*Clostridium dissolvens*).

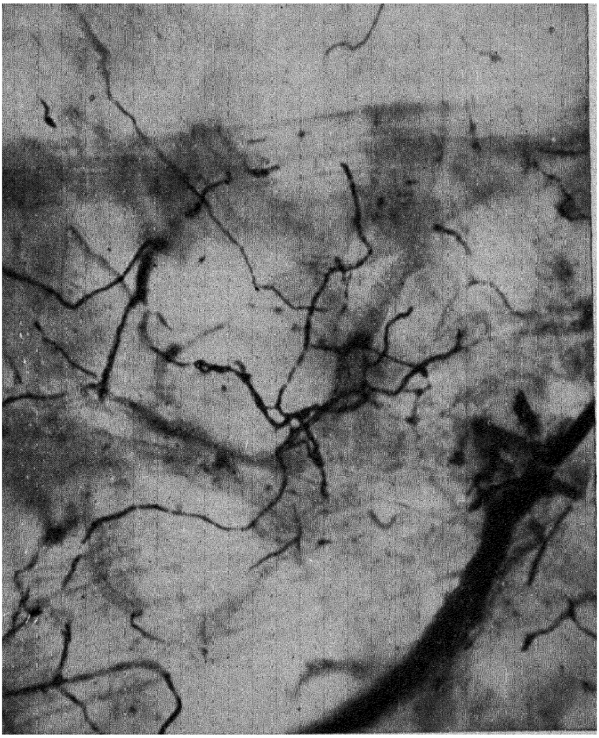


FIG. 173.—Photomicrograph of a cellulose-digesting mold growing on paper. (*Slide prepared by W. H. Swanson.*)

Some thermophilic organisms found in the soil are capable of dissolving cellulose at a temperature of 60 to 65°C. Both aerobic and anaerobic species have been isolated. The anaerobic organisms are easily isolated by inoculating an inorganic medium containing cellulose with an infusion of rapidly decomposing manure and incubating at 65°C. The cellulose is completely dissolved in from 6 to 8 days.

Certain bacteria that reduce nitrates to nitrites are also capable of utilizing cellulose for energy. An inorganic medium containing both

nitrate and cellulose is employed. The decomposition of the cellulose is accompanied by the reduction of the nitrate to nitrite. Gases consisting of a mixture of carbon dioxide and nitrogen are liberated during the reaction.

Most of the organisms that are capable of fermenting cellulose are found in six recognized genera: *Cellulomonas, Clostridium, Cellvibrio, Cellfalcicula, Cytophaga,* and *Actinomyces.*

It is interesting to note that an organism has been isolated from soil capable of liquefying both cellulose and agar. The organism is known as *Vibrio agar-liquefaciens.* It is a motile, short, curved rod. Motility is due to the presence of a single polar flagellum. Surface colonies appear as a whitish growth in a depression surrounded by a white ring. The colony is later surrounded by a ring of liquid agar. Deep colonies show a clear area and may be irregular, oval, or angular. The presence of a readily available carbohydrate prevents the liquefaction of agar.

A considerable number of molds have been described as having the ability to utilize cellulose. The species that have been most extensively investigated are members of the genera *Aspergillus, Penicillium, Trichode ma, Merulius, Paxillus,* and *Polyporus.* Molds are commonly found growing on old paper, especially that kept in a damp atmosphere. They have been responsible for the destruction of many valuable manuscripts (Fig. 173).

For additional information see the review by Boswell (1941).

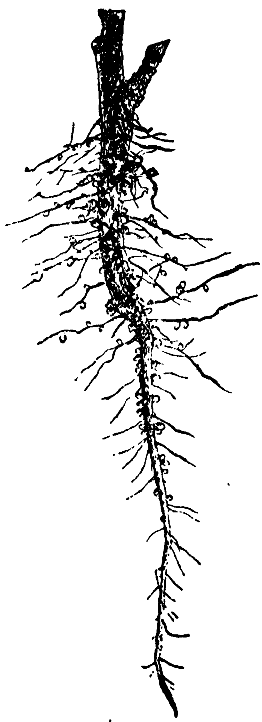


FIG. 174.—Root of *Vicia faba,* showing young nodules on most of the lateral roots. (*After Strasburger; from Lafar's "Handbuch der technischen Mykologie," Gustav Fischer, Jena.*)

SYMBIOTIC NITROGEN FIXATION

It was shown several generations ago that the growth of certain plants in the soil resulted in a stimulation of the succeeding plant crop. The fertility of the soil was greatly increased. The plants responsible for this stimulation were found to be members of the family Leguminosae. Late in the last century it was clearly shown that the stimulation was due to an increase in the nitrogen supply of the soil, which was the result of the presence of small tumor-like growths or nodules on the roots of the leguminous plants. In the absence of nodules no stimulation of growth of the succeeding plant crop occurred.

The formation of nodules on roots is caused by the associated growth of the leguminous plant and a bacterial organism. The bacteria are members of the genus *Rhizobium* and are commonly referred to as the root-nodule bacteria. The organisms live in the cells of the plant roots where their growth and metabolic activities cause a swelling or nodule to form on the root. The organisms utilize the nitrogen of the atmosphere and synthesize it into a nitrogen compound. The plant obtains its nitrogen from the synthetic activities of the organisms while the bacteria derive their food from the plant. The plant and bacteria live together for mutual benefit. Such an association is known as symbiosis.

The organisms responsible for symbiotic nitrogen fixation are members of the genus *Rhizobium*. According to Allen and Allen (1939) the genus is characterized as follows:

Genus: Rhizobium.

Obligate aerobes capable of producing nodules upon the roots of leguminous plants, the symbiosis ordinarily resulting in a fixation of atmospheric nitrogen available to the host plant. Gram-negative rods, 0.5 to 0.9 by 1.2 to 3.0 microns; motile when young, commonly changing to bacteroidal forms upon (*a*) artificial culture media containing alkaloids, glucosides or in which acidity is increased or (*b*) during symbiosis within the nodule. Optimum temperature 25°C. Heterotrophic. Addition of yeast, malt, or plant extracts desirable for rapid growth on artificial media. Slight production of nitrites from nitrates; nitrites not utilized. Gelatin liquefied not at all or only slightly after prolonged incubation.

The genus includes six species. *Rhizobium leguminosarum, R. phaseoli, R. trifolii, R. lupini, R. meliloti,* and *R. japonicum.*

A specific name has not been given to the organism that produces nodules on plants of the so-called "cowpea" group. Walker and Brown (1935) proposed the name *R. japonicum* to include both the cowpea and the soybean groups.

Cross-inoculation Groups.—The seven species are sometimes referred to as cross-inoculation groups. This means that an organism will infect all the plants in a given group but will not attack those in another group. However, there are some exceptions. Occasionally an organism in one group will infect a plant in another cross-inoculation group. No satisfactory explanation has been advanced to account for the above groupings. However, it has been shown that the seed proteins of those plants belonging to the same group are very closely related when tested serologically by the precipitin technique.

The organisms within a given cross-inoculation group do not appear to be all alike. For example, the bacteria from alfalfa and yellow clover are interchangeable but best results are usually produced by inoculating alfalfa plants with alfalfa-specific organisms.

Formation of the Nodule.—The nitrogen-fixing organisms live in the soil in the free state. Recent work points to the fact that they fix nitrogen only when growing in association with the plant. They gain entrance to the plant through the root hairs or other epidermal cells. The bacteria multiply very rapidly, forming long filaments in the root hairs and into the parenchyma of the root. The organisms cause a rapid proliferation of the surrounding tissue in the innermost cells of the root cortex, which results in the formation of a young nodule. The young nodule pushes out the overlying parenchyma and epidermis and produces a swelling on the side of the root. A nodule consists of a mass of thin-walled parenchyma cells, which are usually almost filled with the specific organism. A corky layer and branches of a vascular system are also present. This system provides the bacteria with their nutrients and the plant in turn takes away the nitrogen compounds synthesized by the bacteria.

Plants Involved.—Something like 10,000 species of leguminous plants have been described by botanists. All but a few of them show the presence of root nodules. The nodules vary in size, shape, and position on the different plants. It is generally stated that nodule formation occurs only on plants of the family Leguminosae. It is true that nodule formation occurs most successfully on the Leguminosae but plants of other families are also involved.

Organisms.—Great masses of organisms are present in the nodules. Their presence may be easily demonstrated by crushing a washed nodule between two glass slides, fixing the smear, and staining by the usual technique. It is a relatively simple matter to isolate a pure culture of the organism from a previously washed and sterilized nodule. The usual culture media are not satisfactory for the cultivation of the organisms. A medium that has yielded very good results is known as Ashby's mannitol-phosphate solution. This is an inorganic medium to which is added mannitol as a source of energy. This solution may be solidified by the addition of 1.8 to 2.0 per cent agar. Colonies appear in from 5 to 10 days when the plates are incubated at 25°C.

The colonial characteristics of the organisms show some variation, depending upon the plants from which they are isolated. Some species show large, raised, opaque, and sticky colonies whereas others appear as small, slightly raised, transparent colonies.

The symbiotic, nitrogen-fixing organisms are characterized by the presence of minute rods, which are motile when young. Branching forms are abundant and characteristic in nodules. The organisms are obligate aerobes. Cells from nodules are commonly irregular with X-, Y-, star-, pear-, and club-shaped forms. Swollen or vacuolated

forms appear to predominate. These forms are also known as bacteroids (Fig. 175). Round or coccus cells may also be present. The cocci arise from the swollen or vacuolated cells, which later return to the rod form. Some have described the various forms as orderly stages in the life cycle of the organism.

Although the bacteroid form predominates in the nodule, it is usually absent when nodular material is streaked on agar plates (Figs. 176 and 177). After death and dissolution of the nodule the organisms round up and pass back into the soil in the form of small cocci.

The organisms in the various cross-inoculation groups show some differences in their fermentation reactions. Fermentation usually results in a weak production of acid. This is due to the slow growth rate of the organisms on laboratory media.

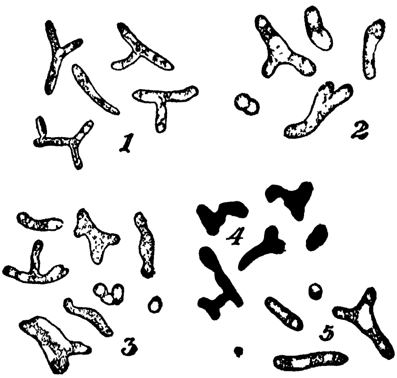


FIG. 175.—Bacteroids from root nodules. 1, *Melilotus alba;* 2, 3, and 5, *Medicago sativa;* 4, *Vicia villosa.* (*After Harrison and Barlow; from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

Some of the root-nodule bacteria show a strong tendency to produce large quantities of gum when grown on carbohydrate media. Gum formation may be so pronounced that the culture becomes very viscous. Organisms in different cross-inoculation groups show great variations in the amount of gum produced. The gum is nitrogen-free, soluble in water, and precipitated from solution on the addition of alcohol or acetone. On being heated with a mineral acid the gum is hydrolized to a reducing sugar.

Mechanism of Nitrogen Fixation.—Several theories have been advanced to explain the mechanism of biological nitrogen fixation but the one proposed by Virtanen and Laine (1939) appears to be the most plausible.

Legumes were inoculated with a root-nodule organism and grown in a sterile, nitrogen-free culture medium. Nitrogen compounds appeared immediately after nodule formation. The nitrogenous compounds were found to be excreted into the soil from the root nodules rather than from the roots. The excreted nitrogen was chiefly amino-N characterized as *l*-aspartic acid. Some β-alanine was also present, which was formed from the *l*-aspartic acid by the legume bacteria. In addition small amounts of oxime-N and nitrite-N were found in the excretion products.

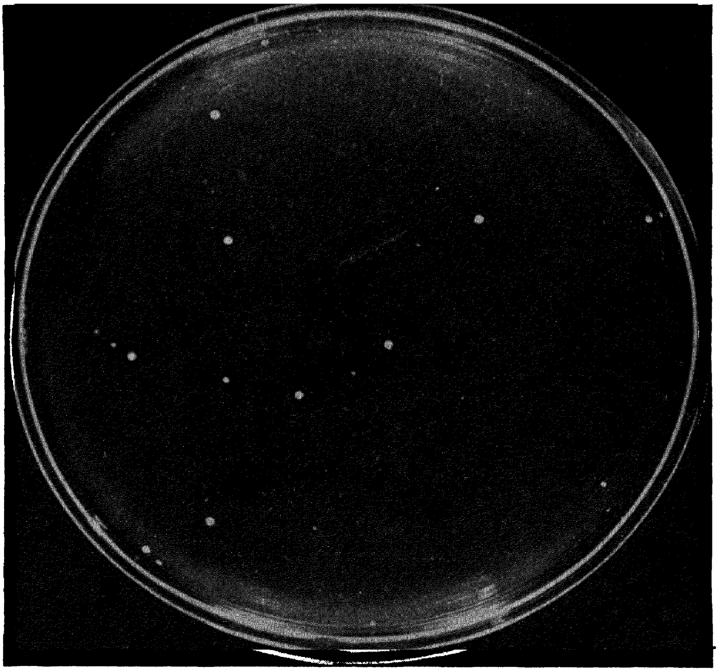


FIG. 176.—Colonies of *Rhizobium leguminosarum* on mannitol agar.

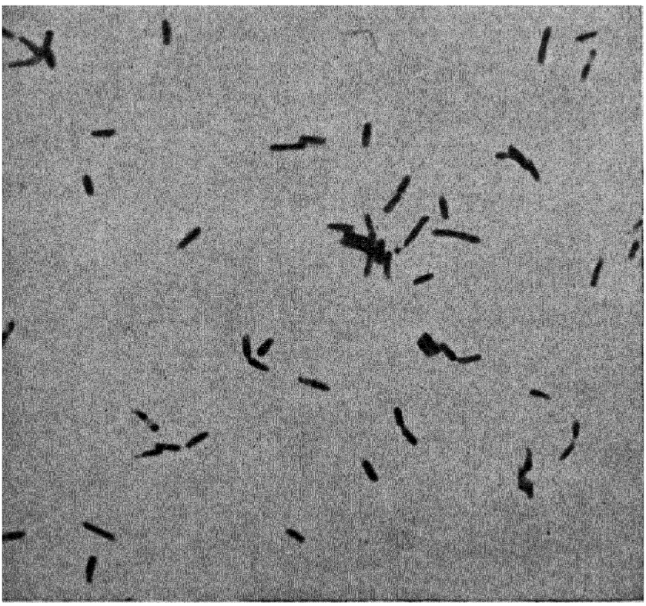
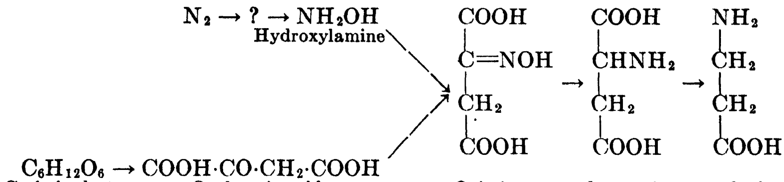


FIG. 177.—*Rhizobium leguminosarum* from culture on mannitol agar.

On the basis of the experimental facts they proposed the following scheme for the biological fixation of nitrogen:



Carbohydrate Oxalacetic acid Oximino-succinic acid *l*-aspartic acid *β*-alanine

From their experimental data, Virtanen and Laine came to the following conclusions:

1. Over 90 per cent of the nitrogen excreted was amino-N. In addition 1 to 2 per cent oxime-N and some nitrite-N was present.
2. The major portion of the amino-N was present as *l*-aspartic acid, if the legumes were harvested at a young stage, long before flowering. The amount of aspartic acid excreted decreased with the age of the plant.
3. *β*-alanine was also found among the excretion products, which was slowly formed from *l*-aspartic acid by the root-nodule bacteria. Its formation explained the decrease of *l*-aspartic acid with age.
4. The oxime was present as oximinosuccinic acid; the nitrite-N was formed from this oxime.
5. A small amount of fumaric acid was also detected among the excretion products.

Artificial Inoculation of Plants.—The efficiency of symbiotic nitrogen fixation has been greatly increased by (1) the choice of the proper legumes, (2) the development of new varieties of leguminous plants, (3) artificial inoculation of seeds with pure cultures of root-nodule bacteria, and (4) the adjustment of the environment to optimum conditions.

Cultures of the organisms have been added to the soil to increase the nitrogen content. This may be practiced either by adding the culture to the soil or by inoculating the seeds with the bacteria. The latter procedure is much easier to carry out and appears to yield superior results. Soaking the seeds in a culture of the specific root-nodule bacteria has become a well-established practice especially when a legume plant is seeded for the first time.

The addition of an abundant supply of nitrates to the soil results in a luxuriant plant growth. Under such conditions nodules probably do not appear on the plant roots, even though the seeds and soil have been inoculated with a pure culture of the specific organisms. The suppression of nodule formation is not due to a direct effect of the nitrate but rather to a change in the metabolism of the plants. On the other hand, the addition of calcium and phosphorus to the soil results in a stimulation of nodule production. Other factors influencing nodule formation include reaction of the soil, temperature, moisture, and oxygen content.

Additional information on symbiotic nitrogen fixation is given in the excellent articles and monographs by Allen and Allen (1936*a*,*b*, 1939), Allison and Minor (1940), Dyal, Smith, and Allison (1939), Fred, Baldwin, and McCoy (1932), West and Wilson (1939, 1940), Wilson (1939, 1940), and Wyss and Wilson (1941).

NONSYMBIOTIC NITROGEN FIXATION

In addition to the organisms discussed in the preceding section, the soil harbors certain nonsymbiotic bacteria that are also capable of fixing

FIG. 178.—*Clostridium butyricum*, showing rods, clostridia, and spores. Nonsymbiotic nitrogen-fixing bacteria. (*After Omelianski, from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

the nitrogen of the atmosphere. The presence of a readily available carbohydrate for energy appears to be necessary for the reaction to take place. Many organisms possess the power to fix nitrogen in small quantities but only a few species are capable of utilizing the gas in relatively large amount.

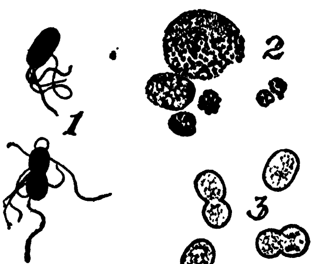


FIG. 179.—Nonsymbiotic nitrogen-fixing bacteria. 1, *Azotobacter agile;* 2, *Azotobacter chroococcum*, degenerate forms; 3, *Azotobacter agile.* (*After Beijerinck, from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

Organisms Concerned.—The first nonsymbiotic, nitrogen-fixing organism isolated was *Clostridium pastorianum* (*butyricum*). It is a motile, Gram-positive, anaerobic, spore-forming rod (Fig. 178). The organism ferments a number of carbohydrates with the production of butyl, ethyl, and isopropyl alcohols, acetone, butyric acid, hydrogen, carbon dioxide, and other compounds. *C. pastorianum* is found in all soils and fixes small amounts of atmospheric nitrogen.

The most important organisms concerned in the fixation of nitrogen nonsymbiotically belong to the genus *Azotobacter*. The organisms appear as relatively large rods, as cocci, and sometimes almost yeast-like (Fig. 179). They obtain their growth energy primarily from the oxidation of carbohydrates. The cells often contain vacuoles and a slimy membrane of variable thickness. Some species are motile, whereas

others are nonmotile. The motile forms show one or a tuft of flagella at one pole. The organisms are obligate aerobes usually growing in a film on the surface of the culture medium. They are capable of fixing nitrogen in a medium containing an available carbohydrate source and deficient in combined nitrogen.

Two species are recognized: *Azotobacter chroococcum* and *A. agile.*

The presence of nitrogen-fixing organisms may be easily demonstrated by inoculating a small amount of fertile soil into an inorganic medium containing glucose. *Clostridium butyricum*, together with other bacteria, will develop in such a solution. If the glucose is replaced by mannitol, organisms belonging to the genus *Azotobacter* will be found to predominate in the culture.

The inoculated medium is best incubated at a temperature of about 25°C. After several days at this temperature a pellicle begins to form on the surface of the medium. The pellicle is at first gray in color, later becoming brownish in appearance. A microscopic examination reveals the presence of many typical cells of *Azotobacter* surrounded by slimy capsules. New cultures are prepared by transferring some of the pellicle to a flask of fresh medium. After several transfers the culture becomes heavily enriched in *Azotobacter.* A loopful of the pellicle is streaked over the surface of a solid medium and incubated. A pure culture is obtained by transferring a typical colony developing on the plate to a tube of fresh medium.

The members of the genus *Azotobacter* are widely distributed in soil. The absence of the organisms from certain soils is due probably to the pH of the soil. *Azotobacter* cells cannot develop if the acidity of the soil is greater than pH6.0. If the reaction is adjusted to the proper pH, the organisms appear in the soil.

The size and shape of the cells depend upon certain environmental factors, such as the composition of the medium, the conditions of cultivation, and the amount of oxygen. The presence of an ample supply of oxygen causes a lengthening of the cells, accompanied by an increase of motility. The addition of colloidal organic substances or aluminum salts to the medium results in the cells remaining young for a long period of time. On the other hand, alkali salts added to the medium stimulate the cells to maturity.

Mechanism of Nitrogen Fixation.—Virtanen and Laine (1939) believed that the reaction mechanism of nitrogen fixation occurring in *Azotobacter* and *Rhizobium* was probably the same. *Azotobacter* and *Rhizobium* showed marked differences in the amounts of their excretion products. The former excreted very small amounts of nitrogen compounds, whereas the latter excreted 60 to 80 per cent of the total fixed nitrogen. The difference in the amounts of excretion products of the

two groups of organisms is probably due to the fact that *Azotobacter* uses the nitrogen compounds largely for its own cell protein, whereas *Rhizobium* excretes most of it into the soil.

Wilson (1939, 1940) demonstrated that molecular hydrogen acted as a specific inhibitor of nitrogen fixation by inoculated, red clover plants. Later Wyss and Wilson (1941) made estimations of nitrogen fixation by three species of *Azotobacter* and obtained essentially the same result. To quote,

> *Azotobacter* cultures grown in an atmosphere in which the pN_2 is reduced to 0.3 atm., the pO_2 kept at 0.2 atm. and the abstracted N_2 either unreplaced or replaced with helium or argon, fix atmospheric N_2 at the same rate as that observed with cultures grown in air. If, however, H_2 is used to replace the N_2, a significant decrease is observed in both rate and extent of fixation. Since the symbiotic nitrogen fixation system of red clover responds to H_2 in the atmosphere in essentially the same manner, it is concluded that the mechanism of nitrogen fixation by the symbiotic system is similar, if not identical, with that of the fixation system in the free-living Azotobacter.

Inoculation of the Soil.—Many attempts have been made to increase the nitrogen content of the soil by inoculation with pure cultures of the specific organisms. In most cases the results have been negative. In a few experiments a detrimental influence was noted. The failures have been attributed to the following factors: (1) absence of a suitable environment, such as proper temperature, moisture, amount of oxygen, food, and reaction of the soil, (2) absence of a source of carbon, and (3) injurious effects due to the end products liberated in the decomposition of added carbohydrate.

The specific organisms are cultivated in the laboratory under optimum conditions for growth. Also, they are grown in pure culture, not in association with other species. When such cultures are introduced into the soil, conditions are encountered that are generally not ideal. This means that most of the organisms soon die. Also, there are so many more bacteria of other genera already in the soil that it is extremely difficult for a laboratory culture to gain a foothold. In order that soil inoculations be successful the chemical, physical, and probably biological conditions of the soil must be made suitable for the growth of *Azotobacter.*

For more information on nonsymbiotic nitrogen fixation consult Burk (1934) and Stephenson (1939).

AUTOTROPHIC BACTERIA

Most bacterial species and other lower forms of life utilize inorganic and complex organic compounds for structure and energy. They are

unable to synthesize carbohydrates, fats, and proteins or their hydrolytic products from water, carbon dioxide, and nitrogen of the atmosphere but must have their food preformed as organic and inorganic compounds. Such organisms are classified under the heterotrophic group.

A few bacterial species are able to obtain their carbon from carbon dioxide and their energy from the oxidation of nitrogen, sulfur, iron, hydrogen, or carbon, either free or in the form of inorganic compounds. These organisms are classified under the autotrophic bacteria.

Higher plants are also able to effect a synthesis of their own organic compounds. They take carbon dioxide from the air and combine it with water to form carbohydrates. Nitrogen is absorbed from the soil in the form of nitrate or other nitrogen-containing compound and synthesized into proteins.

The true autotrophic bacteria show several distinctive physiological characteristics:

1. They grow and multiply in strongly elective mineral media, containing the specific inorganic oxidizable substances.
2. Their existence is dependent upon the presence of these minerals which undergo oxidation as a result of the life processes of the organisms.
3. These oxidations are the only source of energy for the bacteria.
4. The organisms do not require any organic nutrients for either structure or energy.
5. They assimilate carbon dioxide chemosynthetically as the only source of carbon.
6. The presence of small amounts of certain organic compounds may be stimulating at least to some of the autotrophs. Since these organisms are soil inhabitants, they are in practically all cases in contact with soluble organic matter.

The number of obligate autotrophic species is very small. A greater number of species are facultative autotrophs, being capable of existing both autotrophically and heterotrophically. The obligate forms include the nitrifying organisms and some of the sulfur and iron bacteria. The facultative forms can obtain their energy (1) from the oxidation of inorganic substances and the reduction of carbon dioxide for the synthesis of their own protoplasm or (2) from purely organic compounds. Some of the sulfur, iron, and hydrogen bacteria are facultative autotrophs.

The autotrophic group includes forms varying considerably in their morphological appearance. Some of the forms exist as minute cells, greatly resembling in appearance the members of the *Eubacteriales*. Others exist in the form of long filaments. Still others appear to be closely related to the algae in size, shape, and mode of division. Some of the forms contain a pigment known as bacteriopurpurin. This is a red-colored pigment that appears to function in a manner similar to chlorophyll when the bacteria are exposed to the light. The autotrophic bacteria may be classified as follows:

Order I. *Eubacteriales.*[1]
Family 1. *Nitrobacteriaceae.*
A. Bacteria oxidizing ammonia to nitrites.
Genus 1. *Nitrosococcus.*
Genus 2. *Nitrosomonas.*
B. Bacteria oxidizing nitrites to nitrates.
Genus 3. *Nitrobacter.*
C. Bacteria oxidizing sulfur or sulfur compounds.
Genus 4. *Thiobacillus.*
D. Bacteria oxidizing hydrogen to water.
Genus 5. *Hydrogenomonas.*
E. Bacteria oxidizing methane to carbon dioxide and water.
Genus 6. *Methanomonas.*
F. Bacteria oxidizing carbon monoxide to carbon dioxide.
Genus 7. *Carboxydomonas.*
Order II. *Thiobacteriales.*
Family 1. *Rhodobacteriaceae.* Cells not filamentous and contain both sulfur granules and bacteriopurpurin. They are sometimes called the *Thiorhodaceae.*

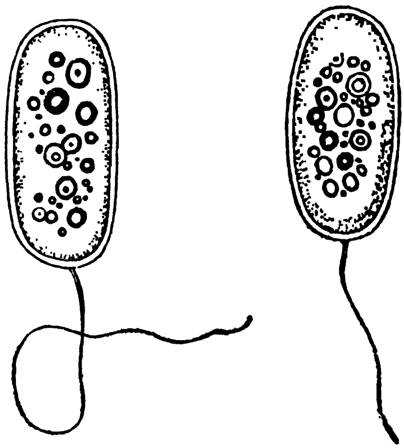


FIG. 180.—*Chromatium okenii.* The round bodies inside of the cells are sulfur granules. (*After F. Forster.*)

Cell division in three directions of space.
Cells capable of swarming.
Genus 1. *Thiocystis.*
Genus 2. *Thiosphaera.*
Genus 3. *Thiosphaerion.*
Cells not capable of swarming.
Genus 4. *Thiocapsa.*
Genus 5. *Thiosarcina.*
Cells united into families. Cell division occurs first in three, then in two directions of space.
Genus 6. *Lamprocystis.*
Cells united into families. Cell division occurs in two planes resulting in the formation of plates of cells.
Cells occurring in a film or membrane.
Genus 7. *Thiopedia.*
Genus 8. *Thioderma.*
Cells occurring in tetrads.
Genus 9. *Lampropedia.*
Cells united into families. Cell division occurs in one direction of space.
Cells connected by plasma threads. Families amoeboid and motile.
Genus 10. *Amoebobacter.*
Cells not like those in the above genus.
Genus 11. *Thiodictyon.*
Genus 12. *Thiothece.*
Genus 13. *Thiopolycoccus.*
Cells not united into families but free and capable of swarming at any time.
Cells elongated and motile.
Genus 14. *Chromatium* (Figs. 180 and 181).
Genus 15. *Rhabdomonas* (Fig. 182).
Genus 16. *Thiospirillum* (Fig. 183).

[1] See Chap. XVI for a description of the various orders, families, and genera.

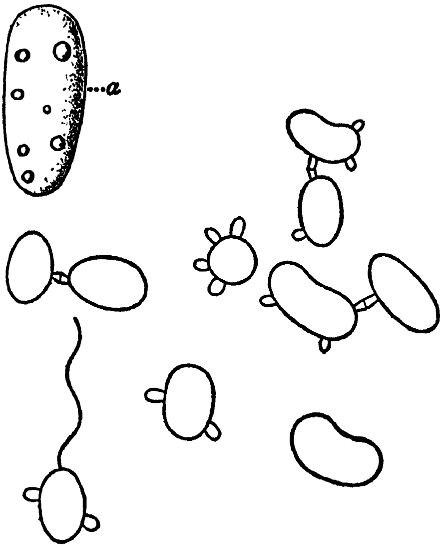


FIG. 181.—*Chromatium minus.* Artificial cultures show the development of buds similar to those observed in yeasts. Motile by means of one large polar flagellum. (*After Bavendamm.*)

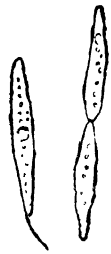


FIG. 182. —*Rhabdomonas rosea.* Spindle-shaped cells. (*After F. Cohn.*)

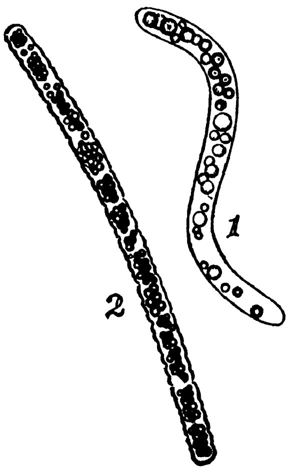


FIG. 183.—1, *Thiospirillum winogradskii.* (*After Omelianski.*) 2, *Beggiatoa alba.* (*After Corsini, from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company, New York.*)

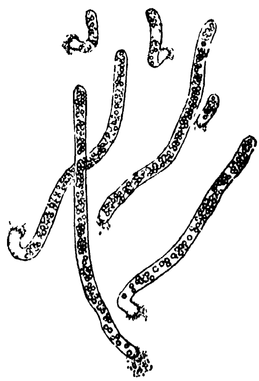


FIG. 184.—*Thiothrix nivea.* Young, unicellular, sulfur-filled filaments with a slime cushion at the base of each. (*After Winogradsky.*)

Cells spherical or slightly elongated and nonmotile.
Genus 17. *Rhodocapsa.*
Genus 18. *Rhodothece.*

Cells not filamentous and contain bacteriopurpurin but no sulfur granules. They are sometimes called the *Athiorhodaceae.*

Cells rod-shaped, clusters embedded in same slimy capsule.
Genus 19. *Rhodocystis.*

Cells spherical or short rods.
Genus 20. *Rhodonostoc.*
Genus 21. *Rhodorrhagus.*

Cells free, elongated but not bent.
Genus 22. *Rhodobacterium.*
Genus 23. *Rhodobacillus.*

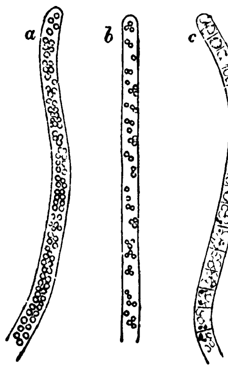


FIG. 185.

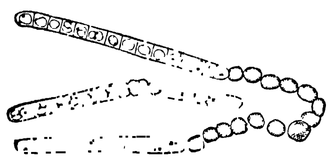


FIG. 186.

FIG. 185.—*Beggiatoa alba.* *a*, filament growing in a medium rich in H_2S; *b*, same filament after 24 hr. in a medium free of H_2S, only a few sulfur granules are present. *c*, same filament after 72 hr. Note the disappearance of the sulfur granules and the appearance of cross walls. (*After Winogradsky.*)

FIG. 186.—*Beggiatoa alba.* Degenerate thread due to lack of H_2S. (*After Winogradsky.*)

Cells free, elongated, and bent or curved.
Genus 24. *Rhodovibrio.*
Genus 25. *Rhodospirillum.*

Family 2. *Beggiatoaceae.* Cells filamentous, nonmotile, differentiated into base and tip.
Genus 26. *Thiothrix* (Fig. 184).

Cells filamentous, motile, not differentiated into base and tip.
Genus 27. *Beggiatoa* (Figs. 185 and 186).
Genus 28. *Thioploca.*

Family 3. *Achromatiaceae.* Cells not filamentous, containing sulfur granules but no bacteriopurpurin.

Cells spherical or ellipsoidal.
Genus 29. *Achromatium* (Fig. 187).
Genus 30. *Thiophysa.*

Cells spiral.
Genus 31. *Thiospira.*

Cells very long and motile.
Genus 32. *Hillhousia.*

Order III. *Chlamydobacteriales.*
Family 1. *Chlamydobacteriaceae.* Bacteria-oxidizing iron compounds.
Genus 1. *Leptothrix.*
Genus 2. *Crenothrix.*
Genus 3. *Sphaerotilus.*
Genus 4. *Clonothrix.*

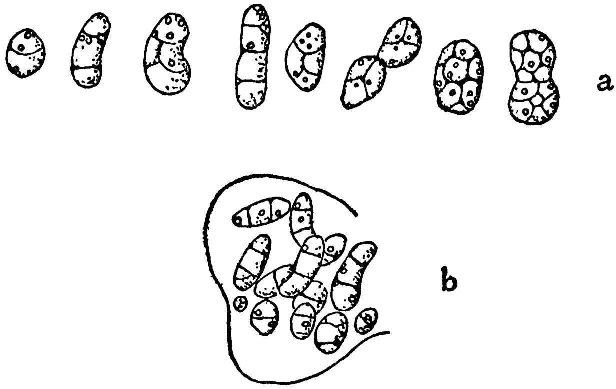


FIG. 187.—*Achromatium oxaliferum.* *a*, zoospores; *b*, zoospores being liberated from mother cell. (*After Virieux.*)

Order IV. *Caulobacteriales.*
Family 1. *Gallionellaceae.* Bacteria-oxidizing iron compounds.
Genus 1. *Gallionella.*

NITROSIFICATION

The organisms responsible for the oxidation of ammonia to nitrite in the soil are members of the genera *Nitrosomonas* and *Nitrosococcus* of the family Nitrobacteriaceae. The species placed in the genus *Nitrosomonas* are *N. europaea*, *N. javanensis*, *N. monocella*, and *N. groningensis*. The bacteria are characterized as rod-shaped cells, motile by means of polar flagella. The rods secure their growth energy by the oxidation of ammonia to nitrite. They grow very poorly or not at all on a medium containing organic matter. The genus *Nitrosococcus* includes only one known species, *N. nitrosus*. The organisms are large spherical cells, showing no growth on ordinary culture media. The species in both genera are aerobic, nonsporulating, and grow best at a temperature of 20 to 25°C.

In order easily to isolate the organisms from soils, conditions must be made unfavorable for the growth of other species except those which are able to oxidize ammonia to nitrite. A simple inorganic medium is employed containing an ammonium compound, magnesium carbonate, and other salts. Since the organisms are strongly aerobic, the cultures should be exposed to the air in shallow layers. Erlenmeyer flasks are suitable for the cultivation of the nitrifiers.

Nitrite formation takes place only in soils having an alkaline reaction. The species of *Nitrosomonas* grow in the pH range of 7.4 to 8.4; the single species of *Nitrosococcus* grows in the range of pH7.0 to 10.3. The ammonia of the medium is oxidized to nitrous acid. As the nitrous acid content increases, the percentage of ammonia decreases. This continues until all of the ammonia has been oxidized to nitrite. The addition of more ammonium salt results in a continuation of the process as long as there is an excess of magnesium carbonate to neutralize the nitrous acid. When all of the carbonate has been decomposed, the organisms should be transferred to a flask of fresh medium.

Colonies may be obtained by streaking the organisms over the surface of an inorganic medium solidified by silicic acid. The inorganic silicic acid is superior to agar for the cultivation of nitrifying organisms. In the presence of insoluble magnesium carbonate clear zones are formed around each colony on the medium, owing to the fact that the magnesium carbonate is dissolved by the acid.

The nitrifiers are widely distributed in nature being present in practically all neutral and alkaline soils. They are the agents primarily responsible for the appearance of nitrate in the soil, which is in turn utilized by higher plants in the synthesis of proteins and other nitrogenous compounds.

NITRIFICATION

Autotrophic organisms that are unable to oxidize ammonia to nitrite but can oxidize nitrite to nitrate are included in the genus *Nitrobacter* under the family Nitrobacteriaceae. The oxidation of nitrite to nitrate is known as nitrification as distinguished from nitrosification.

The medium used for the cultivation of the organisms is similar to that employed in the preceding section except that sodium nitrite is substituted for the ammonium sulfate. The inoculation of such a medium with a suitable soil sample results in a gradual decrease in the nitrite content accompanied by a corresponding increase in the amount of nitrate. Several transfers to fresh medium are necessary to obtain a culture rich in nitrifying organisms. The medium is not specific for the growth of the nitrate-producing organisms alone but may show the presence of other species.

The nitrifiers produce neither pellicle nor uniform turbidity but a slimy layer on the bottom and sides of the flask. A microscopic examination of the slime reveals the presence of minute, spindle-shaped cells, which take the stain with considerable difficulty. The organisms grow on the surface of an inorganic medium solidified with agar. The colonies are very minute, usually brownish, and vary considerably in shape. A pure characteristic colony is transferred to nitrite broth and incubated

at a temperature of 28°C. for several days. The organism is identified by the appearance of nitrate in the medium.

The nitrifying organisms are nonmotile, rod-shaped, obligate aerobic, and nonspore-forming. They grow slowly in organic media. Some species are Gram-positive and some are Gram-negative. The recognized species include *Nitrobacter winogradskyi*, *N. flavum*, *N. agilis*, *N. roseoalbum*, *N. opacum*, and *N. punctatum*. The organisms do not stain uniformly, the central portions taking the color more heavily than the poles.

All neutral or slightly alkaline soils show the presence of organisms capable of oxidizing ammonia to nitrite and finally to nitrate. The optimum reaction appears to be at a pH of from 6.8 to 7.3. The limiting acidity for the development of the organisms in soil is about pH4.0. The addition of an alkalizing chemical, such as lime, to the soil results in the gradual appearance of the specific organisms.

SULFUR CYCLE

Sulfur occurs in the soil in both the free and combined form. In the combined state it exists in the form of both organic and inorganic compounds. It is present in such organic compounds as taurine and its derivatives, in mucin, mucoid, proteins, cystine, cysteine, methionine, methyl mercaptan, etc. The inorganic compounds consist chiefly of sulfates, sulfites, and sulfides.

Sulfur finds its way into the soil from the decomposition of native rock, from organic manures, and from rain water. The gases emitted from volcanoes contain sulfur dioxide and hydrogen sulfide. It is present in large quantities in sulfur springs. Free sulfur is found in the neighborhood of volcanoes, being emitted through these from the interior of the earth. The commercial source of sulfur is chiefly as crude brimstone, obtained from the sides of volcanoes or mined in certain parts of the world. Extensive deposits are found on the island of Sicily and in Louisiana. Commercially it occurs as brimstone or as flowers of sulfur, which is prepared from the crude brimstone.

Sulfur is one of the elements absolutely essential to living organisms. It enters into the composition of all plant and animal cells. The addition of sulfur to a soil low in this element results in a marked stimulation of growth. As the plants and animals die and decompose, the sulfur finds its way into the atmosphere. Volcanoes emit large quantities of sulfur dioxide and hydrogen sulfide. The burning of coal also releases gaseous compounds of sulfur. The gases are dissolved by rain water and again returned to the soil. Some soil organisms are able to convert the sulfur-containing gases to sulfates. Some of the sulfate is utilized by growing plants and some is leached out by waters and carried off to the ocean.

The sulfate in the ocean may be reduced to sulfide and then precipitated by iron as iron sulfide or it may be converted into insoluble calcium sulfate. Deposits of gypsum (calcium sulfate) are believed to have been formed in this manner. Deposits of sulfur probably resulted from the reduction of sulfate to sulfite and then to free sulfur, or from the oxidation

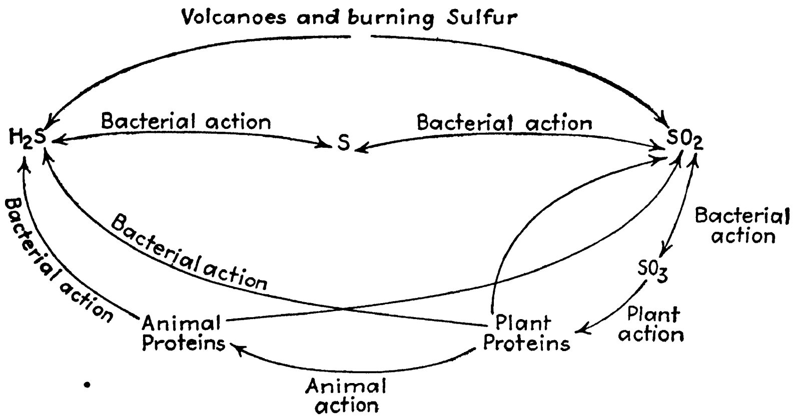


FIG. 188.—The sulfur cycle.

of hydrogen sulfide. The sulfur cycle may be diagramed as shown in Fig. 188.

THIOBACILLUS

The members of the genus *Thiobacillus* are small, rod-shaped organisms capable of deriving their energy from the oxidation of sulfides, thiosulfates, or free sulfur, forming sulfur, persulfates, and sulfates under acid or alkaline conditions. They obtain their carbon from carbon dioxide or from bicarbonates and carbonates in solution. With one exception all species are aerobic. Some of the aerobic species are obligate and some are facultative autotrophic. Eight species are recognized. Two important members are *Thiobacillus thioparus* and *T. thiooxidans*.

Thiobacillus thioparus.—In an inorganic medium containing sodium thiosulfate the organisms are capable of oxidizing the compound with the formation of sulfates and free sulfur. The free sulfur is further oxidized to sulfuric acid.

$$5Na_2S_2O_3 + H_2O + 4O_2 \rightarrow 5Na_2SO_4 + H_2SO_4 + 4S$$
$$4S + 6O_2 + 4H_2O \rightarrow 4H_2SO_4$$

A pellicle, consisting of a mixture of bacteria and sulfur granules, is produced on the surface of the medium in from 24 to 48 hr. The addition of agar to the same medium, followed by inoculation, results in the appearance of colonies from which a pure culture may be obtained.

In the presence of air containing carbon dioxide, but no dissolved carbonate or bicarbonate, growth proceeds at a much slower rate. In the absence of both carbon dioxide and dissolved carbonate and bicarbonate no growth takes place even in the presence of various organic compounds. The organisms are unable to accumulate sulfur within their cells, although an abundant production takes place outside of the cells. The production of sulfur and sulfate from thiosulfate is an exothermic reaction resulting in the release of considerable energy. The energy is utilized for the reduction of sodium bicarbonate and for the synthesis of compounds for structure. The sodium thiosulfate may be replaced by a metallic sulfide, tetrathionate, or hydrogen sulfide. Carbon dioxide cannot be replaced by organic carbon compounds as a source of carbon. Agar colonies of the organisms are very sensitive to artificial conditions of cultivation and die after a period of about one week.

Thiobacillus thiooxidans.—The cells are characterized as short rods with rounded ends, occurring singly and in short chains. The rods are nonmotile. The organisms grow best in an acid environment. The cells use carbon dioxide as a source of carbon. Bicarbonates are utilized only in small amounts and carbonates not at all.

The addition of sulfur to soil results in an accumulation of sulfuric acid.

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4$$

If powdered insoluble calcium phosphate is added to a mixture of sulfur and soil, it is transformed into soluble phosphate by the sulfuric acid formed from the oxidation of the sulfur.

$$Ca_3(PO_4)_2 + 3H_2SO_4 + 6H_2O \rightarrow 3CaSO_4{\cdot}2H_2O + 2H_3PO_4$$

The tricalcium phosphate is converted to phosphoric acid. The amount of phosphate dissolved is directly proportional to the sulfuric acid content of the soil.

The rods are easily cultivated in an inorganic salt medium without carbonate, and having free sulfur and an acid reaction. Since the organism is obligately aerobic, the exposure of the medium in flat, shallow layers hastens the growth of the rods. A microscopic examination of the sulfur granules shows them to be surrounded by the specific organisms. At the same time there is a great increase in the acidity of the medium. The organism grows in the pH range of 1.0 to 6.0 with an optimum at 2.0 to 4.0. The presence of a calcium salt in the medium results in the precipitation of insoluble calcium sulfate. *T. thiooxidans* produces more acid than any living organism yet reported. The hydrogen-ion concentration of the medium rises to a pH of 0.6 or less.

HIGHER SULFUR BACTERIA

The higher sulfur bacteria are members of the order Thiobacteriales, which includes forms intermediate between the true bacteria and the blue-green algae. They contain either granules of free sulfur, or bacteriopurpurin, or both and usually grow best in the presence of hydrogen sulfide. The cells are plant-like, not protozoan-like. Spores are rarely or never produced.

The organisms are commonly present in water containing dissolved hydrogen sulfide. The gas results from the decomposition of organic matter by saprophytic organisms. The bacteria may be present in such organic materials as decomposing seaweed, in rock pools containing dead algae and other lower forms of plant life, in stagnant woodland pools, in sewage, etc. Sulphur bacteria have been found in hot sulfur springs and in sulfur mines. Some species are able to grow in water pipes and cause serious obstructions. Foul odors and tastes are produced after death and decomposition of the organisms. A large number of the colored organisms growing together cause a body of water to appear red or purple.

Bacterial Photosynthesis.—The pigmented, higher sulfur bacteria are generally divided into two groups on the basis of their nutritional requirements: (1) the Thiorhodaceae, comprising the green and purple sulfur bacteria that develop in a mineral medium in the presence of hydrogen sulfide and (2) the Athiorhodaceae, embracing the purple bacteria that require organic substances for growth.

The most important contributions on the photosynthetic activities of the higher sulfur bacteria have been reported by van Niel (1935, 1936*a*,*b*). The experimental results of his studies can be summarized as follows:

Thiorhodaceae.—1. Bacteria exist that can develop in inorganic media containing hydrogen sulfide, in the presence of sunlight, and under completely anaerobic conditions.

2. These organisms fail to develop in the absence of hydrogen sulfide.

3. In a mineral medium containing sodium bicarbonate, ammonia nitrogen, potassium, phosphorus, and magnesium, the amount of growth is proportional to the concentration of hydrogen sulfide present.

4. Growth of the organisms fails to occur in the absence of carbon dioxide (carbonate, bicarbonate).

5. Oxygen is not produced.

6. During growth of the green sulfur bacteria hydrogen sulfide is oxidized to free sulfur which is deposited outside of the cell.

$$CO_2 + 2H_2S \xrightarrow{+\text{ light}} CH_2O + H_2O + 2S$$

In the case of the purple sulfur bacteria the H_2S becomes oxidized to sulfuric acid,

$$2CO_2 + H_2S + 2H_2O \xrightarrow{+\text{ light}} 2CH_2O + H_2SO_4$$

7. The reaction of the medium becomes more and more alkaline due to utilization of the carbon dioxide.

8. A stoichiometrical relationship exists between the amount of hydrogen sulfide oxidized and the quantity of carbon dioxide that disappears. For the green sulfur bacteria 0.5 mole of carbon dioxide disappears to each mole of hydrogen sulfide oxidized to sulfur; for the purple sulfur bacteria almost 2 moles of carbon dioxide disappears to each mole of hydrogen sulfide oxidized to sulfuric acid.

9. The carbon of the carbon dioxide that disappears can be recovered from the bacterial bodies as organic carbon.

10. Growth does not occur in the dark even though the environment is anaerobic. The hydrogen sulfide is not oxidized to sulfur or to sulfuric acid and carbon dioxide does not disappear.

The biological conversion of carbon dioxide into organic matter in the presence of light is known as photosynthesis. Since the green and purple sulfur bacteria (Thiorhodaceae) are capable of producing this reaction, they are considered to be photosynthetic organisms.

The photosynthetic reaction is believed to be as follows:

$$CO_2 + H_2S \xrightarrow{+\text{ light}} CH_2O + H_2O + 2S$$

in which the hydrogen sulfide acts as a hydrogen donor.

The photosynthetic bacteria have been cultivated in media containing simple organic compounds instead of hydrogen sulfide or other sulfur compounds. If the organic compound employed is represented by the formula H_2A the reaction now becomes,

$$CO_2 + 2H_2A \xrightarrow{+\text{ light}} CH_2O + H_2O + 2A$$

in which the organic compound serves as a hydrogen donor to reduce the carbon dioxide.

Athiorhodaceae.—The members of the Athiorhodaceae also contain bacteriopurpurin and are light-sensitive under anaerobic conditions, but they show the following dissimilarities to the Thiorhodaceae:

1. They do not contain sulfur granules within their cells.

2. It is generally stated that they develop only in the presence of organic matter, but this is not strictly correct.

Carbon dioxide can be reduced by organic compounds instead of by hydrogen sulfide (Gaffron 1933, 1935). In the presence of butyric acid the reaction becomes

$$CO_2 + C_3H_7COOH + H_2O \xrightarrow{+\text{ light}} 5CH_2O$$

The Athiorhodaceae are also capable of assimilating carbon dioxide in the presence of hydrogen, according to the reaction,

$$CO_2 + 2H_2 \xrightarrow{+\text{ light}} CH_2O + H_2O$$

Approximately two molecules of hydrogen react with one molecule of carbon dioxide. In addition, the organisms utilize hydrogen sulfide photosynthetically in the presence of organic matter and carbon dioxide.

From the foregoing it may be concluded that the two groups of organisms are photosynthetic only in the absence of free oxygen and require unusual hydrogen donors for the photochemical reduction of carbon dioxide. The general reaction

$$CO_2 + 2H_2A \xrightarrow{+\text{ light}} CH_2O + H_2O + 2A$$

may be used to express the metabolism of both groups of organisms.

For more information on bacterial photosynthesis consult the reports of van Niel (1935, 1936*a*,*b*, and 1941).

IRON BACTERIA

Iron is, next to oxygen, the most abundant element necessary for living cells. In some species only minute amounts have been detected, but, even if the quantities required are small, the element is absolutely necessary for the growth and well-being of all plants and animals.

Magnesium, rather than iron, is present in chlorophyll, the green coloring matter of plants. However, in the complete absence of iron, leaves do not become green. Also, if iron is withheld from a plant in which the chlorophyll is well developed, the color gradually fades to yellow.

Certain microorganisms present in water and soil are capable of taking up iron and accumulating it on the surfaces of their cells where it quickly oxidizes to ferric hydroxide. The organisms are known as the iron bacteria. The cells are classified under the orders Chlamydobacteriales and Caulobacteriales, which include those organisms having characteristics intermediate between the true bacteria and higher plants.

The autotrophic, iron bacteria are capable of oxidizing ferrous to ferric compounds and the energy so obtained is utilized for the chemosynthetic assimilation of carbon. These organisms should be distinguished from the bacteria that are able to absorb or accumulate iron when grown in ferruginous liquids or cause a precipitation of iron, owing to changes in the hydrogen-ion concentration of the medium.

The reaction for the oxidation of iron is believed to be as follows:

$$4FeCO_3 + 6H_2O + O_2 \rightarrow 4Fe(OH)_3 + 4CO_2$$

Ferruginous waters usually show the presence of a yellowish- or reddish-colored slime on the stream bottom. The color is due to the deposi-

tion of iron in the outer sheath of the filaments. The accumulation of iron and its oxidation to ferric hydroxide results in the formation of a hard and inelastic membrane, which eventually leads to the death of the organisms. Old filaments show a higher iron content than do young filaments. In some cases young cells are completely lacking in a deposition of iron in their sheaths. The iron hydroxide may be removed by the application of dilute hydrochloric acid, after which the outer membrane becomes visible.

Five genera of iron bacteria are recognized: (1) *Sphaerotilus,* (2) *Clonothrix,* (3) *Leptothrix,* (4) *Crenothrix,* and (5) *Gallionella.* The first

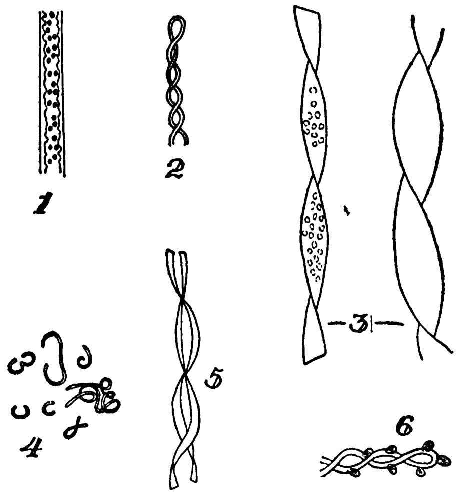


FIG. 189.—Iron bacteria. 1, *Leptothrix ochracea*; 2, 3, 4, 5, 6, *Gallionella ferruginea.* (*After Ellis, from Lipman's "Bacteria in Relation to Country Life," The Macmillan Company.*)

four are members of the order Chlamydobacteriales, whereas the last one is placed under the Caulobacteriales.

Sphaerotilus.—The organisms are attached, colorless threads, which show false branching. The filaments consist of rod- or oval-shaped cells surrounded by a thin sheath. The sheath is composed entirely of a deposit of colloidal ferric hydroxide. Multiplication occurs through the formation of conidia within the sheath of the vegetative cells. The conidia swarm at one end, float about for a time, and finally attach themselves to solid objects where they develop into delicate filaments. The motile cells have a tuft of flagella near one end.

Clonothrix.—The filaments are attached and show false branching as in the genus *Sphaerotilus.* The base of a filament is broader and tapers toward the tip. A sheath is always present and later becomes encrusted with a deposit of iron. The cells are colorless and cylindrical. Mul-

tiplication takes place by means of small, nonmotile, spherical conidia. They result from the disk-shaped cells near the tip by longitudinal division and rounding up of the contents.

Leptothrix.—The filaments are composed of cylindrical, colorless cells, surrounded by a sheath which is at first thin and colorless, later becoming

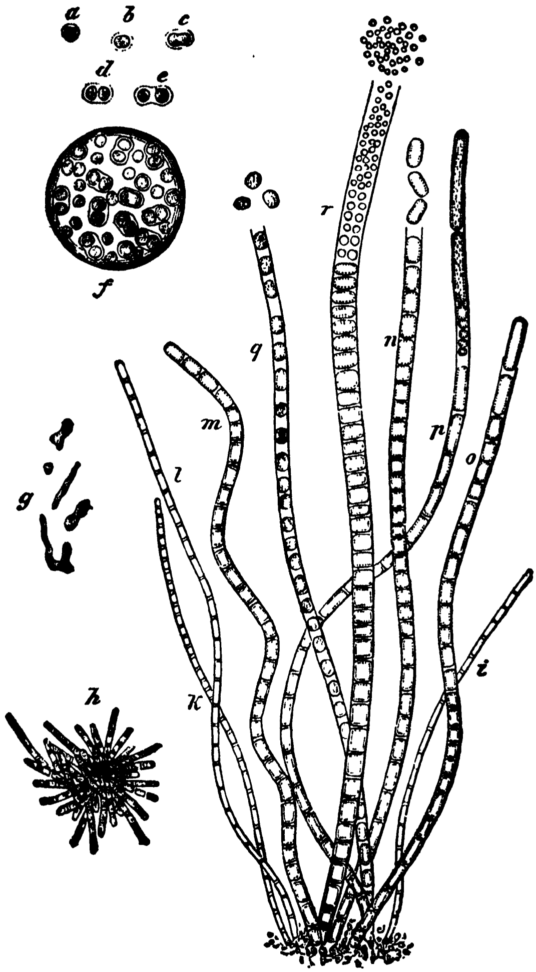


FIG. 190.—*Crenothrix polyspora.* *a–e*, reproduction of the cocci; *f*, zoogleal mass of cocci; *g*, same as *f*, natural size; *h*, same as *f*, beginning to germinate; *i–r*, threads of different diameter. (*After Zopf.*)

thicker and yellow or brown in color. The filaments are surrounded with iron oxide. The iron is easily dissolved by dilute hydrochloric acid, exposing the inner cells. Multiplication results by the division and abstraction of cells and by motile, cylindrical, swarm cells. Swarm cells sometimes germinate in the sheath, giving the filaments the appear-

ance of true branching. The organisms are found in rivers, lakes, ponds, and swamp waters containing iron in combination with organic matter. The best known species, *L. ochracea*, is world-wide in distribution (Fig. 189).

Crenothrix.—Filaments are unbranched, attached to a firm substrate, and show no differentiation of base and tip. Sheaths are easily seen, being thin and colorless at the tip, and thicker and encrusted with a deposit of iron at the base. The cells are cylindrical to spherical in shape, dividing in three planes to produce spherical nonmotile conidia. The conidia may escape and germinate into new cells or germination may occur inside of the sheath. *C. polyspora* is the only species recognized (Fig. 190).

The organisms are found in stagnant and running water containing organic matter and iron salts. The organisms grow in thick masses imparting a brownish or greenish color to the water. They do not grow on artificial media. When the filaments die they are rapidly decomposed by saprophytic organisms present in water, resulting in the liberation of bad odors.

Gallionella.—This genus includes some of the stalked bacteria. The long axis of the rod-shaped cells is set at right angles to the axis of the stalk. The cells occur in the form of filaments, twisted singly or two together (Fig. 189). Young cells are colorless, later becoming brown to rust red through the accumulation of iron. The twisted filaments are easily identified since no other organism of a similar character has ever been observed to take such a form. When the loops of the coils become encrusted with a deposition of iron, the filament resembles a row of beads. The presence of a sheath has not been demonstrated. The organisms attach themselves to pipes and cause extensive deposits of iron, which seriously interfere with the flow of water. For this reason they are sometimes known as the water-pest bacteria. The organisms are widely distributed in nature.

For an excellent discussion of the iron bacteria consult the monograph by Cholodny (1926).

PHOSPHORUS CYCLE

Phosphorus is another element that is never found in nature in the free state. It is a constituent of many inorganic and organic compounds, being present in phosphates, nucleic acids, lecithins, phytin, coenzymes, etc. Phosphorus is found in the soil and in natural deposits. In both instances it is present chiefly as phosphates. It is present in the protoplasm of nearly all cells as a constituent of nucleic acids. The framework of man and animals consists largely of calcium phosphate.

Lecithin.—Many soil organisms are capable of attacking organic phosphorus compounds. Lecithin is hydrolyzed with the liberation of glycerol, fatty acids, phosphoric acid, and choline. Many lecithins are possible, depending upon the nature of the fatty acid radicals present (see page 241).

Phytin.—Phytin is the phosphoric acid ester of inositol. It occurs abundantly in vegetable tissues, especially in seeds, and grains. The enzyme phytase which is elaborated by some organisms, is capable of hydrolyzing phytin to inositol and phosphoric acid (see page 242). The inositol is attacked and fermented like other carbohydrates or carbohydrate-like compounds.

Calcium Phosphates.—Insoluble calcium phosphates are converted into soluble phosphates by soil organisms. The solvent action is believed to be due to the presence of both organic acid and carbon dioxide formed by soil bacteria. Carbon dioxide appears to be more efficient than the organic acids for solubilizing the insoluble phosphates.

$$Ca_3(PO_4)_2 + 4H_2O + 4CO_2 \rightarrow Ca(H_2PO_4)_2 + 2Ca(HCO_3)_2$$
$$2CaHPO_4{\cdot}2H_2O + 2CO_2 \rightarrow Ca(H_2PO_4)_2 + Ca(HCO_3)_2 + 2H_2O$$
$$Ca_4P_2O_9 + 5H_2O + 6CO_2 \rightarrow Ca(H_2PO_4)_2 + 3Ca(HCO_3)_2$$

The various organic acids include butyric, lactic, acetic, citric, oxalic, fumaric, etc. These acids react with the phosphates to give butyrates, lactates, acetates, citrates, oxalates, fumarates, and phosphoric acid. The salts of the organic acids are usually further oxidized to carbon dioxide and carbonates.

The presence of ammonium salts in the soil results in their oxidation to nitrous and nitric acids by the autotrophic bacteria. The acid corresponding to the negative radical will also be released in the soil. If the salt is ammonium sulfate, some sulfuric acid will be liberated. The acids are capable of reacting with insoluble calcium phosphate, converting it into soluble compounds. The reactions may be represented as follows:

$$Ca_3(PO_4)_2 + 2HNO_2 \rightarrow 2CaHPO_4 + Ca(NO_2)_2$$
$$Ca_3(PO_4)_2 + 4HNO_2 \rightarrow Ca(H_2PO_4)_2 + 2Ca(NO_2)_2$$
$$Ca_3(PO_4)_2 + H_2SO_4 \rightarrow 2CaHPO_4 + CaSO_4$$

A relatively high concentration of hydrogen ions is required to convert insoluble tricalcium phosphate into the soluble form. Most of the liberated nitrous acid reacts with calcium and magnesium carbonates and salts of organic acids. This means that the nitrous acid is replaced by weak, organic acids. In the presence of considerable acid some insoluble phosphate is dissolved. However, the degree of acidity required for this purpose is so high that it would probably produce a destructive effect on plant growth. Therefore, the nitrifying bacteria are responsible for only a small amount of the soluble phosphate in the soil.

Sulfur is oxidized to sulfuric acid by members of the genus *Thiobacillus.* The dissolution of rock phosphate by sulfuric acid is very similar to its transformation by nitrous acid. The sulfuric acid reacts with calcium and magnesium carbonates and salts of organic acids in preference to insoluble phosphates. The high acidity (about pH3.0) required to dissolve phosphates is distinctly injurious to growing plants. The phosphorus cycle may be diagramed as shown in Fig. 191.

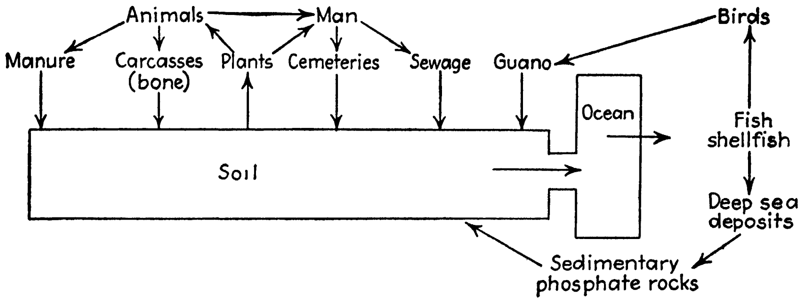


FIG. 191.—The phosphorus cycle. (*After Lotka.*)

Nucleoproteins.—Nucleoproteins are found widely distributed in plants and animals, being present in nearly all cells. They occur chiefly in the nucleuses of cells. Nucleoproteins are compounds of protein combined with nucleic acid. Different proteins and nucleic acids have been isolated—indicating that many kinds of nucleoproteins occur in nature. The proteins present are basic in character, being members of the groups known as the protamines and the histones.

Nucleoproteins give an acid reaction and are insoluble in water. They are soluble in weak alkali but are precipitated from solution on the

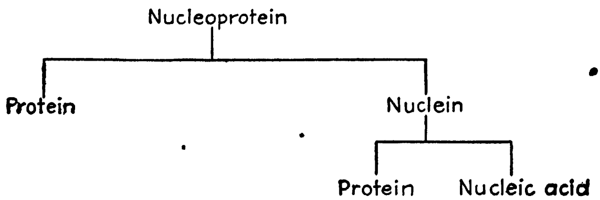


FIG. 192.—The hydrolysis of a nucleoprotein to nucleic acid and protein.

addition of acid. Nucleoproteins are very complex in composition and are unstable chemically. In many cases they appear to be mixtures of protein and nucleic acid rather than definite chemical compounds. The action of enzymes or weak acid on nucleoproteins results in a splitting off of some of the protein transforming the compound into a mixture of protein and nuclein. The nuclein still contains some protein. More prolonged enzymatic action or treatment with acid removes the remainder of the protein, setting free nucleic acid. This may be expressed diagrammatically as shown in Fig. 192.

Nucleic Acids.—Nucleic acids are strongly acid in reaction and contain considerable amounts of phosphorus. They may be divided into two types: (1) animal nucleic acids and (2) plant nucleic acids. On hydrolysis both types yield four molecules of phosphoric acid, four molecules of carbohydrate, two molecules of purines, and two molecules of pyrimidines. Plant nucleic acids contain the pentose *d*-ribose, whereas animal nucleic acids contain the desoxypentose *d*-2-ribodesose. The purines adenine and guanine are present in both types of nucleic acids.

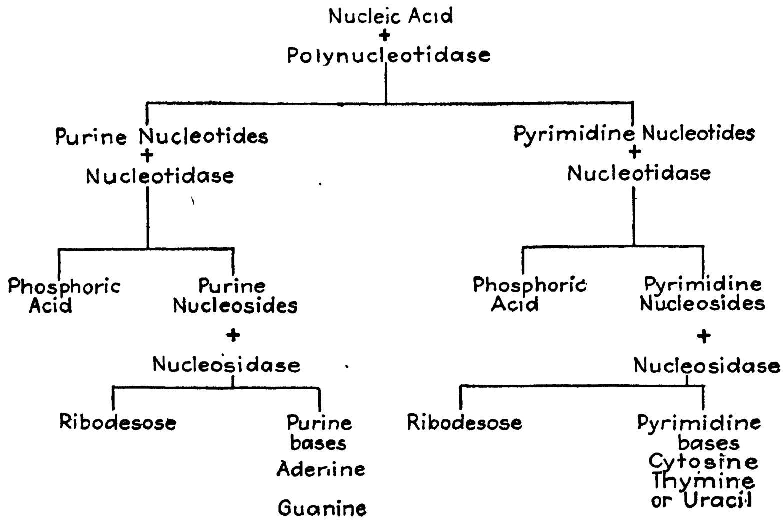
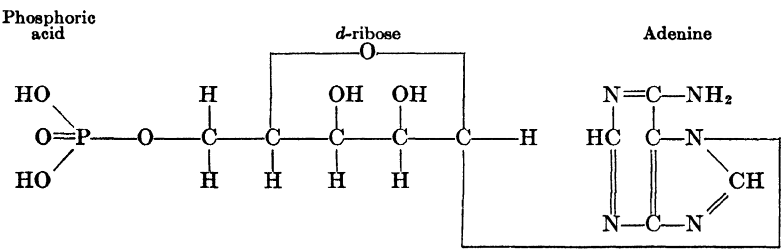


FIG. 193.—The enzymatic hydrolysis of animal nucleic acid to its constituent units.

They differ again in the kinds of pyrimidines present. Plant nucleic acids contain cytosine and uracil whereas animal nucleic acids contain thymine and cytosine. A nucleic acid molecule is known as a tetranucleotide, being composed of four mononucleotides.

The structure of a mononucleotide is as follows:



Adenylic acid

The above mononucleotide yields phosphoric acid, *d*-ribose, and adenine on hydrolysis. The nucleotide takes its name from the purine or pyrimidine base present. For example, a nucleotide containing

guanine is known as guanylic acid, one containing cytosine is called cytidylic acid, etc. A nucleic acid molecule is composed of four mononucleotides joined together by ester linkages of the phosphoric acid with secondary alcohol groups of the sugar.

The nucleic acid molecule is hydrolyzed to its constituent units by enzymatic action. The hydrolysis of animal nucleic acid, including the enzymes concerned in the reactions, is diagramed in Fig. 193.

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CHAPTER XXIV

INFECTION AND IMMUNITY

Infection.—The term infection may be defined as the entrance, growth, and multiplication of organisms in the body, resulting in the development of a disease process.

Contamination.—The terms infection and contamination are not synonymous and should be distinguished from each other. A contaminated object is one that contains bacteria, especially those capable of producing disease. A drinking cup may be contaminated with the organism of tuberculosis, but it is not infected. Likewise, the hands may be contaminated with disease bacteria without being infected.

Bacteria are found in various parts of the body. Their presence does not mean necessarily that a disease process has been established. They are normally present on the skin, in the mouth, in the nasal passages, in the upper respiratory tract, on the conjunctiva, in the intestinal tract, etc. Some species are constantly present in each locality. Staphylococci and streptococci are present on the skin, the colon organism (*E. coli*) is found in the intestinal tract, *Corynebacterium xerose* is present on the conjunctiva, streptococci and pneumococci are found in the normal mouth, etc.

Incubation Period.—The incubation period of a disease may be defined as the interval of time between the entrance of a disease organism into a host and the first appearance of symptoms. This period may be a few hours, several days, often weeks, and even months. For typhoid fever it is usually about 10 days. Even though the length of the incubation period may vary, depending upon the virulence of the organisms, it can never be entirely eliminated. Just what happens during the incubation period is not clearly understood in every case. In rabies the length of the incubation period is a measure of the time required for the virus to reach the central nervous system. The farther away from the central nervous system the bite from a rabid animal is located, the longer will be the incubation period.

Communicable Disease.—A communicable disease may be defined as one that is transferred naturally from one individual to another. All communicable diseases are infections produced by microscopic organisms, the causative agents of which are transferred from one person to another by contact, by coughing and sneezing, and in other ways. An infectious

disease is not necessarily a communicable disease. For example, lockjaw, or tetanus, is an infectious disease but not communicable in the true sense of the term. The organism produces an infection of wounds and has no natural means of reaching a similar wound in another individual. However, the great majority of infectious diseases are communicable. This includes such diseases as whooping cough (pertussis), mumps (parotitis), measles (rubeola), smallpox, influenza, etc. The most infectious communicable diseases are those which attack the upper respiratory tract, being usually transferred during coughing, sneezing, and talking.

Endemic, Epidemic, Pandemic Disease.—An endemic disease may be defined as one that occurs constantly among the population of a community. An epidemic disease is one in which a large number of cases develop in a community within a short time. A pandemic disease is an epidemic disease of wide distribution. The widespread occurrence of influenza during 1917–1919 was at first epidemic and then became pandemic, spreading over the entire world.

Pathogenicity.—A pathogenic organism is one capable of producing a disease. Thousands of bacterial species have been isolated but only a few of these are capable of producing disease in man. Some are pathogenic for man but not for animals. Conversely, some species produce disease in animals but fail to do so in man. The ability of an organism to invade and produce a disease process depends upon the species concerned. Therefore, it is necessary to name the host in order that the term pathogenic may be used correctly.

Saprophyte and Parasite.—Bacteria may be classed as saprophytes or parasites. The saprophytes are those organisms which live best on dead organic matter. They are the forms that are of great economic importance in reducing complex dead organic matter into simple soluble compounds, which may again be available to plants and bacteria. Parasitic organisms, on the other hand, are those which find conditions favorable to their growth in living bodies of man and animals. This latter group includes the disease-producing or pathogenic organisms.

Some exceptions have been noted. Occasionally a saprophytic organism becomes pathogenic, resulting in the establishment of a disease process. This is especially true when the defense mechanism of the host is reduced following a severe illness. Also, not all parasites are pathogenic. The colon bacillus (*E. coli*) lives normally as a harmless parasite in the intestinal tract but only rarely invades the tissues to produce an infection.

Virulence.—Virulence may be defined as the degree of invasiveness of a pathogenic organism. Different strains of the same species may show great variability in their invasive powers. Also, the same strain

kept under different conditions may show great differences in disease-producing ability.

As a rule, a pathogenic organism decreases in virulence when transferred from its natural environment to artificial culture media. Other unfavorable environmental conditions also reduce the virulence of a pathogenic species. A strain that has been greatly reduced in virulence is said to be attenuated. Some organisms, like the pneumococcus, may lose their virulence entirely when transferred to culture media. Such a culture is said to be nonvirulent or avirulent. Other organisms retain their virulence even though cultivated on culture media for many generations.

Various methods are employed for decreasing the virulence of an organism. In addition to the use of culture media for this purpose, animal passage may be employed. For example, cowpox virus is smallpox virus that has been reduced in virulence for human beings by cultivation in the tissues of the cow.

An organism that is attenuated by passage through one animal species may be increased in virulence by passage through another species. For example, the virulence of the pneumococcus may be greatly increased by passage through white mice. In this way a strain that was originally avirulent for mice may become so stepped-up in virulence that 0.000001 cc. of a broth culture of the organisms will kill a mouse in 48 hr.

Number of Organisms.—The number of organisms plays a very important part in determining whether or not an infection will occur. A small number of virulent pathogenic organisms may be easily attacked and destroyed, whereas a larger number may not be completely eliminated by the defense mechanisms of the host. This explains why some individuals are attacked by an organism even though they have been previously immunized against the same species. The immunization will take care of a few invaders but breaks down when a mass attack occurs. In general, the number of organisms required to produce disease is inversely proportional to their virulence.

Path of Infection.—Bacteria gain entrance to the body in various ways. Some enter through the broken skin (occasionally through the unbroken skin), some by way of the respiratory passages, others by way of the alimentary tract. The portal of entry determines whether or not pathogenic bacteria are capable of producing an infection. The organism of typhoid fever, if rubbed into the broken skin, would probably not produce an infection but, if swallowed, may reach the intestinal tract and produce the disease. The organism of gas gangrene will have no effect if swallowed but, if rubbed into the broken skin, may result in a fatal infection.

Therefore, bacteria must enter the body by the route to which they are adapted. However, this is not the only factor that determines that infection will result. Man and animals possess several defense mechanisms for destroying invading bacteria. If these mechanisms are vigorous and very active, they will usually defend the host against the disease organisms. On the other hand, if they are below normal and the invaders are very virulent, an infection may take place.

After bacteria invade the tissues, they may attack the host in a variety of ways. The organisms may produce a local inflammation or may localize in the liver, bone marrow, spleen, lymph glands, etc., giving rise to secondary abscesses or secondary foci of infection, also known as metastatic infections. Sometimes organisms invade the blood stream, producing a bacteremia or septicemia (blood poisoning).

Bacterial Waste Products.—Bacteria produce a large number of waste products in the culture medium in which they are growing. The formation of some of these compounds is dependent upon the presence of certain specific precursors in the culture medium. The formation of others is not dependent upon the composition of the medium but is a characteristic of the organisms themselves. The composition of the medium merely determines whether the compounds shall be produced in greater or smaller amounts.

To the former group belong such compounds as the ptomaines (amines), indole, skatole, phenol, and hydrogen sulfide. Specific amino acids must be present in the peptone of the medium; otherwise these compounds will not be formed. The latter group includes the true bacterial toxins. These are of two kinds; the exotoxins and the endotoxins. The exotoxins are elaborated by the bacterial cells and secreted into the surrounding culture medium. These may be recovered by passing the culture through an appropriate filter, which removes the bacterial bodies from the medium. Only a few pathogenic bacteria are capable of secreting true soluble toxins of great potency. The symptoms produced are due largely to the toxins secreted by these organisms. In other words, the injection of the cell-free filtrate produces symptoms characteristic of the disease. The best-known members of this group are *Corynebacterium diphtheriae*, *Clostridium tetani*, *Clostridium botulinum*, types *A*, *B*, and *C*, and some of the sporulating anaerobes isolated from cases of gas gangrene. The endotoxins, on the other hand, are not secreted into the surrounding culture medium but remain confined within the bacterial cells. They are released only after the death and dissolution of the organisms. Most organisms fall in this group. An example is the organism of typhoid fever. If a young culture of this organism is filtered, the filtrate will show only a slight toxic effect, whereas

the organisms themselves may produce a very poisonous action. Filtrates of old cultures may be highly toxic, owing to the death and autolysis of the organisms resulting in the liberation of the endotoxins.

Some organisms have been shown to elaborate both exotoxins and endotoxins. The bacteria of cholera and dysentery appear to belong to this group, although they elaborate considerably more endotoxin than exotoxin.

The exotoxins appear to be quite thermolabile, being destroyed by moderate heating. They are easily decomposed on standing in the presence of oxygen (air). They are believed to be protein in nature, although their chemical composition has not been determined. The endotoxins show greater resistance to adverse conditions than the exotoxins.

Mode of Action of Bacterial Toxins.—Bacterial toxins exhibit their characteristic action by producing a specific effect on some organ or tissue. The toxins of *Clostridium botulinum* and *Clostridium tetani* have been shown to exhibit a selective affinity for the nervous system. Some organisms, notably the hemolytic streptococci, attack and dissolve red blood corpuscles. Other organisms exhibit a selective action on the white blood cells or leucocytes. It is believed that the toxin produces a physical or chemical union or both with the specific tissue or organ involved. It has been shown that bacterial toxins, when injected into animals, rapidly disappear from the blood stream, indicating that a union with the specific tissue has taken place.

Resistance.—The fact that bacteria enter the body through the mouth, the nasal passages, or a break in the skin does not mean than an infection will take place. If this were true, man would have disappeared from the earth long ago. The power of the animal body to prevent growth and development of organisms after they have gained entrance is spoken of as resistance. The various defense mechanisms come into play and in most cases quickly remove the invading bacteria. Sometimes the resistance to a disease is characteristic of a species. It is then spoken of as immunity.

Various degrees of immunity have been shown to exist. One race may be immune to a certain disease; another is susceptible. This does not mean that the former race cannot be given the disease. Small doses of the organisms may be easily disposed of, but massive doses are usually able to overcome the natural defenses of the host with the result that disease develops. Chickens are immune to anthrax because their body temperature is too high for the growth of the organism. If the body temperature is reduced to 37°C., chickens become susceptible to the disease.

NATURAL IMMUNITY

A race or species may inherit a resistance to a certain infectious disease. This resistance is spoken of as natural immunity.

Species Immunity.—Many of the organisms that attack humans do not attack animals. Typhoid fever infections do not occur in animals except after massive experimental inoculations with the specific organism. Human leprosy has never been transmitted to animals successfully. Meningitis does not occur spontaneously in animals but may be produced experimentally. Many of the animal diseases do not occur spontaneously in man.

It is not known why differences in species susceptibility exist. It may be due to differences in temperature, metabolism, diet, etc. Diseases of warm-blooded animals cannot be transmitted to cold-blooded animals and vice versa.

Racial Immunity.—The various races also exhibit differences in their resistance to disease. Many examples may be mentioned. Negroes and American Indians are considerably more susceptible to tuberculosis than the white race. It is known that exposure during a period when the disease is endemic results in the development of a more resistant race to that disease. On the other hand, negroes are more resistant to yellow fever and malaria than the white race.

Individual Immunity.—Laboratory animals of the same species, kept under the same environmental conditions, exhibit only slight differences in their resistance or susceptibility to experimental disease. On the other hand, higher animals show wide differences in susceptibility to disease. For example, during an epidemic of influenza there are always some individuals who do not contract the disease even though in close contact with the virus. These individuals exhibit a higher degree of resistance than do the majority of the members of the race. Also, those who contract the disease show great variation in the severity of the symptoms.

ACQUIRED IMMUNITY

An individual of a susceptible species may acquire a resistance to an infectious disease either accidentally or artificially. This resistance is spoken of as acquired immunity.

Accidental.—Many of the infectious diseases, such as typhoid fever, scarlet fever, and measles, usually occur only once in the same individual. The resistance of the host to the disease is increased so that another exposure to the same specific organism usually produces no effect. This resistance or immunity may last for a limited time or for life.

Artificial.—Immunity may be acquired artificially by means of vaccines or by the use of immune serums. If the immunity is acquired by

means of vaccines, it is spoken of as active immunity; if it is acquired by the use of immune serums, it is spoken of as passive immunity.

Active Immunity.—Active, artificial immunity may be produced in a variety of ways. It may be produced by (1) a sublethal dose of a virulent organism, (2) a sublethal dose of dead bacteria (vaccine), (3) an injection of an attenuated (reduced virulence) culture, (4) immunization with bacterial products (exotoxin).

1. Sublethal Dose of Virulent Organisms. This method has been practiced by some investigators. It has been employed experimentally with the organism causing cholera. It is not applicable to very virulent organisms, such as *B. anthracis*, the causative agent of anthrax.

2. Sublethal Dose of Dead Bacteria (Vaccine). Active immunization by this method is practiced to a considerable extent against those organisms which produce only small amounts of soluble toxins (exotoxins). The method has been used successfully against typhoid fever, the paratyphoid fevers, cholera, and to a lesser degree against staphylococcus and streptococcus infections.

The organisms to be used for immunization are grown on an appropriate solid medium or in broth. If the organisms are grown on a solid medium, they are removed and suspended in salt solution. The broth culture of the organisms or the suspension in salt solution is sterilized by the application of heat, or by the addition of an appropriate germicide. Portions of the suspension are then transferred to media to test the vaccine for sterility. Both aerobic and anaerobic cultures are prepared. If the vaccine is sterile, it is standardized to contain a definite number of organisms per cubic centimeter. This will vary depending upon the organism. *Staphylococcus aureus* vaccine is usually standardized to contain 500 million to 1 billion organisms per cubic centimeter. Typhoid vaccine usually contains 1 billion organisms per cubic centimeter. A germicide, such as tricresol or phenol, is added to preserve the product and measured amounts are distributed in sterile vials or bottles.

A vaccine, prepared from organisms grown on a solid medium, contains only the bacterial antigen in suspension. A vaccine prepared from a broth culture contains not only bacterial bodies but also various excretory products of the organisms. If the organism elaborates an extracellular toxin, the broth culture vaccine will be more valuable as an immunizing agent than one prepared from the growth on a solid medium. Immune bodies will be developed against both extracellular toxin and bacterial protein.

Vaccines prepared from laboratory stock cultures are known as stock vaccines. Various kinds of stock vaccines are prepared and may be purchased.

Vaccines prepared from two or more species are referred to as mixed vaccines. Probably the most commonly employed vaccine of this type is the common cold vaccine. A typical vaccine for colds and respiratory diseases in general has the following formula:

	Million
Hemophilus influenzae	300
Streptococcus (several kinds)	300
Diplococcus pneumoniae (all types)	300
Klebsiella pneumoniae	300
Micrococcus catarrhalis	200
Staphylococcus aureus	500
Staphylococcus albus	500
Total per cubic centimeter	2400

Vaccines prepared from organisms freshly isolated from the patient to be treated are called autogenous vaccines. Such vaccines have been shown to be superior to those prepared from stock cultures because various strains of the same species may show some variation in antigenicity. Also freshly isolated organisms are considerably more virulent than those carried on stock cultures.

The vaccines already discussed consist of dead bacteria in suspension. They are sometimes, and probably more correctly, referred to as bacterins. In a more restricted sense the term vaccine is applied only to those preparations containing living organisms but it has now taken on a broader meaning to include biologicals containing both dead and living organisms. The two most important vaccines containing living organisms (viruses) are smallpox and rabies vaccines.

3. Injection of an Attenuated Culture. Pathogenic organisms rapidly lose their virulence by transfer on artificial culture media. Repeated passage through some animals increases the virulence, whereas the reverse effect may occur by passage through another animal species.

Pasteur, Chamberland, and Roux (1881) reduced the virulence of the anthrax organisms by cultivation at a temperature of 42°C. instead of at 37°C. Although the attenuated organisms were not capable of producing anthrax, they were satisfactory for immunization against the disease. Chamberland and Roux (1883) showed that the same result was achieved by growing the organisms in the presence of a dilute germicide (phenol 1:500). The bacteria lost their ability to produce spores (asporogenous) and became avirulent for sheep. However, the organisms were very satisfactory for purposes of immunization.

4. Immunization with Bacterial Products. Some organisms excrete soluble compounds into the surrounding culture medium known as extracellular toxins or exotoxins. They may be recovered in an impure state by centrifugation of the culture followed by filtration through an appropriate filter to remove the living bacterial cells.

The symptoms produced by such organisms are largely the result of the action of the soluble products elaborated by the bacterial cells. Immunization against organisms of this group occurs following injection of gradually increasing doses of the filtrate or toxin. This method is followed in producing antitoxins against diphtheria, tetanus, botulinus, and the organisms responsible for gas gangrene. Unfortunately only a few organisms are capable of excreting a potent extracellular toxin.

Passive Immunity.—It is evident that in active immunization a certain period of time is necessary before the cells of the host elaborate sufficient antisubstances to be of definite value in the prevention of disease. The method is of value before symptoms of the disease appear. It is essentially a prophylactic treatment. Under some conditions it may be used to incite antibody formation in certain chronic diseases.

In passive immunity, on the other hand, a temporary immunity may be acquired by injecting into the body an immune serum obtained from an immune animal or man. The protection enjoyed is due to the substances transferred to the patient, which are present in the immune serum. This type of immunization is practically limited to those diseases caused by organisms that elaborate powerful exotoxins. The active immunization of an animal following the injection of several doses of an exotoxin gives rise to an immune substance known as an antitoxin. The best representatives of this group are diphtheria and tetanus antitoxins, which have been of tremendous importance therapeutically. However, immune serums prepared against organisms that do not produce exotoxins are of value in some diseases. The antisubstances present in the serum of an animal injected with a suspension of bacteria (vaccine) are directed against the proteins of the bacterial bodies. The immune substances possess the power to attack and dissolve the specific bacteria used in their production. These are known as antibacterial serums as distinguished from antitoxic serums.

Antitoxins are of great value prophylactically and especially after symptoms of disease have appeared. However, passive immunity lasts for only a relatively short period of time. It has been shown that antitoxin injected into a man becomes less and less from day to day and may be expected to disappear from the blood within a period of about two weeks. Antibacterial serums are employed before, at the same time that, or soon after, infection takes place. Most of them are ineffectual and of minor importance therapeutically.

ANTIGENS

An antigen may be defined as any substance that, when introduced into the tissues, will stimulate the formation of antibodies. Sometimes the antibodies remain attached to the cells producing them, in which case

they cannot be demonstrated in the blood. However, the antibodies are usually found in the circulating blood and may be easily recognized by appropriate tests.

A substance, in order to be antigenic, must be foreign to the species producing the antibodies. This means that the injection of guinea pig serum into a guinea pig will not produce antisubstances, but the injection of guinea pig serum into a rabbit results in a vigorous antibody response.

Nature of Antigens.—Antigens are, with very few exceptions, protein in nature. Some workers believe that all antigens are proteins. Others have brought forward evidence to show that there might be a few exceptions. Carbohydrates and other nonprotein compounds are usually not antigenic but, when combined with protein, they may determine the specific character of the antibody that the whole compound produces, and will react only with that antibody. A substance of this type is known as a partial antigen or hapten.

On the other hand, not all proteins are antigenic. In order that a protein be capable of inciting the production of antibodies it must be soluble in blood plasma. Unless this occurs, it cannot reach the site of antibody formation. Proteins that have been irreversibly coagulated by heat usually fail to exhibit antigenic activity. There does not appear to be any relationship between protein toxicity and antigenic activity. Many proteins are nontoxic and yet elicit strong antibody responses.

It is not known why some proteins are antigenic and others are not. Gelatin is an example of a protein that is not antigenic. It is known that gelatin is lacking in aromatic amino acids, *i.e.*, acids having benzene rings. It contains no tryptophane nor tyrosine and only a trace of phenylalanine. Some have concluded from this observation that the presence of aromatic acids is necessary for a protein to exhibit antigenic properties.

Molecular Size of Antigens.—Antigenic proteins are colloidal in nature. This means that they are composed of large molecules. Diffusible compounds having relatively small molecular weights do not incite antibody formation because they readily pass into cells and are easily attacked and destroyed. An antigenic protein, when hydrolyzed to small units, such as peptones, peptids, and amino acids, becomes nonantigenic.

TOXINS AND ANTITOXINS

Antitoxins may be defined as immune bodies elaborated by living cells following the injection of bacterial filtrates containing soluble toxins. The bacterial bodies play no part in the reaction except to elaborate the soluble antigenic substances.

The injection of specific antitoxin into a patient suffering from a disease caused by a toxin-producing organism results in the neutralization of the toxin. The toxin is neutralized by the specific antitoxin without the destruction of either. The two components may be dissociated by acids, by high dilution, and in other ways. Several theories have been advanced to explain the mechanics of the reaction.

Arrhenius (1907) attempted to explain the neutralization of toxin by antitoxin on purely chemical grounds analogous to a reaction between a weak acid and base to give a salt. He believed that an equilibrium was reached in which there was present free toxin and antitoxin as well as some combined toxin-antitoxin, the quantities depending upon the relative proportions of each in the mixture, according to the mass-action law.

$$\text{Toxin } (T) + \text{antitoxin } (AT) \rightleftharpoons \text{toxin-antitoxin } (TAT)$$

$$\frac{(T)(AT)}{TAT} = K$$

Bordet (1909), on the other hand, believed that the reaction could be explained as an adsorption phenomenon. He believed that the toxin and antitoxin did not unite in definite proportions similar to a chemical reaction but that the neutralization was purely physical. One serious objection to the adsorption theory is that it fails to explain the specificity of the reaction. Some believe that the neutralization is neither entirely physical nor entirely chemical but a combination of both theories.

AGGLUTINATION AND PRECIPITATION

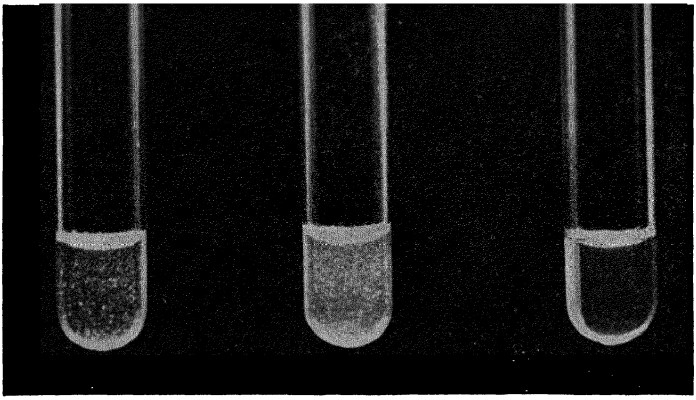
Gruber and Durham (1896) noticed that when bacteria (antigen) were mixed with specific antiserum, a gathering together or clumping of the organisms occurred. The clumping was due to the presence, in the immune serum, of antibodies known as agglutinins. The antigens (bacterial suspension) are spoken of as agglutinogens (Fig. 194).

Later Kraus (1897) observed that bacterial filtrates produce precipitates when mixed with specific immune serum. The precipitating antibodies in immune serum are spoken of as precipitins and the soluble antigens as precipitinogens (Fig. 195).

It is believed that agglutination and precipitation are produced by the same antibodies. In one case (agglutination), the antigen consists of particulate matter (bacteria or other cells); in the other (precipitation), the antigen is in solution. The two immune substances may be demonstrated in the same antiserum. For example, a typhoid antiserum will not only agglutinate typhoid organisms but will also produce precipitates with the culture filtrates. This holds true regardless of whether the

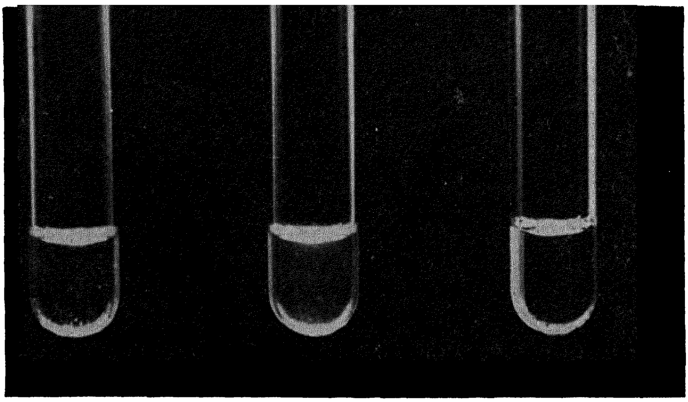
serum was removed from an animal previously immunized against typhoid organisms or a culture filtrate.

Agglutination and precipitation are similar to the neutralization of toxin by antitoxin in that only two components are concerned in the reaction. These are the antigen (agglutinogen or precipitinogen) and the immune substances (agglutinins or precipitins).



A B C

FIG. 194.—Agglutination. Suspension of typhoid organisms mixed with typhoid antiserum. *A*, complete agglutination; *B*, partial agglutination; *C*, control.



A B C

FIG. 195.—Precipitation. Horse serum mixed with horse antiserum. *A*, complete precipitation; *B*, partial precipitation; *C*, control.

Normal Agglutinins and Precipitins.—Normal serums of man and animals often possess the power to precipitate or agglutinate the specific antigen, provided the serums are not too highly diluted. It is not known if these normal agglutinins and precipitins are the same as the corresponding immune bodies, *i.e.*, those obtained following immunization.

Formalin (solution of formaldehyde), heat, ultraviolet rays, chemicals, etc., in concentrations just strong enough to kill bacteria do not destroy the agglutinating antibodies. However, most immune serums lose their agglutinating ability at a temperature of 60 to 65°C.

Presence of Electrolytes.—Bordet (1899) first noted that agglutination did not occur in the complete absence of an electrolyte, such as sodium chloride. However, the addition of only a minute amount of electrolyte to a nonagglutinating mixture of antigen and agglutinin caused agglutination to take place at once. This was shown to apply not only to bacteria, but to other cells. Agglutination is believed to proceed in two steps. First, the antibody becomes fixed to the antigen, and second the cells clump together in the presence of an electrolyte. The reaction is analogous to the precipitation of colloids by electrolytes. The union of antigen and antibody leads to the formation of an amphoteric colloidal suspension that is easily precipitable by electrolytes. Only minute amounts of electrolytes are required.

Hardy (1900) was the first to point out that the precipitation of colloidal particles is determined by the ion of the added electrolyte having an electrical charge opposite in sign to that of the colloidal particles. Since bacteria carry a negative electrical charge, the positively charged ions of the electrolyte will condition the agglutination of the organisms. The greater the positive charge the smaller will be the quantity of electrolyte required to cause agglutination.

LYTIC PHENOMENA

Nuttall (1888) found that when normal blood was mixed with tissue containing anthrax organisms and incubated for a definite period of time, a reduction in the number of bacteria occurred. In other words, fresh normal blood exerted a bactericidal action. Buchner (1889*a*,*b*) observed that the bactericidal action of normal blood serum was destroyed by heating to a temperature of 56°C. for 30 min. He named this substance alexin (a defense substance that destroys bacteria) because of its resemblance to enzymes. Later, Pfeiffer and Issaeff (1894*a*,*b*) observed that if cholera organisms were injected into the peritoneal cavity of a guinea pig (previously immunized against the same organism) they lost their motility, broke up into granules, and finally dissolved completely. They also showed that the injection of a mixture of cholera organisms and immune serum into a normal guinea pig resulted in a dissolution of the bacteria (bacteriolysis). Bordet observed that the reaction took place not only in the presence of unheated immune serum, but also in the presence of immune serum previously heated to 55°C. for 30 min. and mixed with a small amount of normal serum. These observations showed that two factors are concerned in the phenomenon of bacteriolysis:

(1) a thermolabile substance present in normal and immune blood serum and (2) the immune substances that are produced during the process of immunization. The immune bodies are specific, whereas the thermolabile substance is not specific.

Specific, lytic, immune serums may be prepared not only against bacteria (bacteriolysins) but also against other cellular bodies such as red blood corpuscles (hemolysins), and tissue cells (cytolysins).

If a bacterial serum is heated to 55°C. for 30 min., it will be unable to dissolve the specific antigen. However, the addition of a small amount of normal serum restores the bacteriolytic power of the immune serum. This reaction shows that the thermolabile substance, known as complement or alexin, is present in all animal serums and that it is not increased in amount by immunization. The other component (known as immune body, immune substance, amboceptor, and sensitizer) is not destroyed when the serum is heated to 55°C. for 30 min. and is increased in amount by immunization.

This group of antibodies requires three components for action: antigen, antibody, and complement. The antibody first reacts with the antigen. In the absence of complement no dissolution of the antigen takes place, but in the presence of this component the cells are dissolved. On the other hand, agglutination, precipitation, and antitoxic immune reactions require only two components: antigen and antibody.

Complement Fixation.—This is the name given to an immunological reaction that was first demonstrated by Bordet and Gengou (1901) and is of considerable importance in bacteriology. They noted that when the serum of a patient who had recovered from bubonic plague was mixed with the specific antigen (plague bacilli), a binding or fixing of the complement of the serum occurred, resulting in a dissolution of the organisms. Since no free complement remained in the serum, they called the reaction "complement fixation." It is sometimes referred to as the Bordet-Gengou reaction.

Briefly, the test is as follows:

1. Antigen and antibody must first unite before dissolution of the cells by complement can occur.

2. The antigen is bound only by the specific antibody.

3. If the antigen and antibody are bound (sensitized antigen), the complement of the serum becomes fixed.

4. The absence of free complement in the serum indicates that it has been fixed by the antigen-antibody union.

5. The presence or absence of free complement in the serum may be detected by adding a mixture of sheep red corpuscles and sheep immune serum (previously heated to 55°C. for 30 min. to destroy complement) to the antigen-antibody mixture. In the presence of free complement the

sheep cells will undergo dissolution; in its absence no hemolysis will occur, indicating that the complement has been bound by the antigen-antibody complex. The test is of value in diagnosing the presence of certain disease organisms in patients suffering from disease or for the identification of certain antigens by means of specific serums.

The test, as performed by Bordet and Gengou, may be outlined as follows:

A	*B*
Antigen (Suspension of plague organisms) + Antibody (Plague-immune serum heated to 55°C. for 30 min.) + Complement (Fresh normal serum)	Antigen (Suspension of plague organisms) + Normal serum (Heated to 55°C. for 30 min.) + Complement (Fresh normal serum)

To both *A* and *B* after 5 hr. was added:
Sheep red-cell-immune serum
(Heated to 55°C. for 30 min.)
+
Sheep red blood cells

Results:

A. Hemolysis did not occur.
B. Hemolysis occurred.

WASSERMANN REACTION

This reaction is of great importance in the diagnosis of syphilis. The test is similar to the complement-fixation reaction of Bordet and Gengou with one important exception. The antigen is not composed of specific, antigenic protein as is used in the complement-fixation reaction but consists of lipoidal suspensions. These lipoids are obtained from various sources and bear no relation to the disease or to the organism of syphilis. The antigen is composed of a mixture of lipoidal substances including lecithin and cholesterol. Only colloidal suspensions containing lipoidal material are effective.

The lipoidal suspension is not an antigen in the true sense. It is not capable of inciting the production of immune bodies when injected into an animal. It resembles an antigen in that it is capable of binding syphilitic antibodies in the serum of the patient and for that reason is generally spoken of as an antigen. In Wassermann's original method an extract of syphilitic tissue was used as the antigen. This type of antigen

is no longer used, having been replaced by various colloidal suspensions of lipoidal material.

The Wassermann test is sometimes positive in conditions other than syphilis, such as malaria, tuberculosis, and leprosy, and under some conditions may be negative in syphilitic individuals. In general, however, the test gives reliable results and, when used in connection with clinical findings and history, is of great importance as an aid to diagnosis and treatment. The test is complicated and subject to errors in the hands of the inexperienced worker.

Kahn Test.—The antigen employed in the Kahn test is an alcoholic extract of dried beef heart, prepared after previous ether extraction, and cholesterinized by the addition of 6 mg. of cholesterol to 1 cc. of the extract. It is a specially prepared, concentrated antigen containing the colloidal material in larger, unstable particles.

In the Wassermann reaction a mixture of antigen and syphilitic serum does not give a visible precipitate. When the Kahn antigen is mixed with syphilitic serum, a visible precipitation occurs.

Kline Test.—The antigen employed in the Kline test is made from the alcohol-soluble, acetone-insoluble portion of beef heart extract, concentrated by evaporation at 50°C.

The Kline test is demonstrated as a microscopic-slide reaction. One-half cubic centimeter of a syphilitic serum is placed on a slide and mixed with one drop of the antigen. The slide is rotated for 4 min. at a speed of about 100 rotations per minute and then examined under the low-power objective of the microscope. A negative test shows a homogeneous emulsion without the presence of clumps.

The Kahn and Kline tests are simpler to perform and not subject to so many errors as is the Wassermann reaction. Also, the results may be obtained more quickly. These two tests are being slowly adopted in place of the Wassermann reaction and it appears to be only a question of time until they will replace the older method entirely.

ANAPHYLAXIS

The term allergy includes all types of reactions of hypersensitiveness to foreign substances regardless of whether they are antigenic or non-antigenic. The term anaphylaxis is generally employed in a loose sense. Strictly speaking, it is defined as a hypersensitiveness to substances that are antigenic, *i.e.*, capable of stimulating the production of antibodies when injected.

A guinea pig may be inoculated with a large dose of foreign antigen without any ill effects being noted. The animal is then said to be sensitized. If, after an incubation period of 10 days or more, the animal is given a minute injection of the same antigen (intoxicating dose),

violent symptoms may occur, leading to the death of the animal. If the animal recovers, it is usually refractory to another injection of the same antigen. If a guinea pig is given repeated injections of a foreign protein, at brief intervals of 7 days or less, it does not become hypersensitive and usually shows only a mild reaction. The animal is then said to be desensitized to further injections of the same antigen.

All animal species are not equally susceptible to anaphylaxis. Guinea pigs are more sensitive than any other laboratory animal. Death is due to a bronchial spasm followed by acute asphyxia and death. Man, on the other hand, usually shows only skin lesions (rash).

The immune bodies responsible for anaphylaxis are probably identical with precipitin and complement-fixing antibodies. Anaphylaxis is believed to be a cellular reaction, taking place within the cells in which the antibody is fixed. Some of the antibodies are released from the cells and may be demonstrated in the blood stream.

The symptoms are due to a contraction of the smooth or nonstriated muscle tissue. This may be shown by removing a piece of smooth muscle from a sensitized guinea pig and placing it in a solution of the specific antigen. Contraction immediately takes place.

Skin Test for Allergy.—Skin tests may be used to determine if an individual is sensitive to certain foods, pollens, or other proteins. The test is performed by introducing extracts of the various protein substances into the skin of the arm and noting the results. A positive test is indicated by the appearance of a large, localized, inflamed area, or urticarial wheal, surrounding the site of injection. Many different extracts may be tested at the same time, and the reaction appears after a few minutes.

For more information see the review by Dragstedt (1941).

PHAGOCYTOSIS

The immunological phenomena already discussed in this chapter dealt almost entirely with reactions between bacteria, bacterial excretory products (toxins), and other antigens with body fluids. These were the first immunological reactions recognized. It was subsequently shown by Metchnikoff (1901) and others that certain body cells also play a part in the defense mechanisms of the host.

The cells that are chiefly concerned in the reaction are the white blood corpuscles known as the polymorphonuclear leucocytes or phagocytes. These cells are capable of wandering to the site of infection, of engulfing bacteria, tissue fragments, etc., and of removing them from the infected area. Since the cells chiefly concerned in the reaction are known as phagocytes, the process is generally referred to as phagocytosis. The leucocytes are sometimes called scavenger cells because they clear

away bacteria and debris. Many of the leucocytes are destroyed in their attempt to remove the invading bacteria.

The power of phagocytosis is not limited to the polymorphonuclear leucocytes but is possessed by a number of other cells, some of which are fixed tissue cells, and some of which are wandering cells. The former includes the endothelial cells that line the capillaries and sinuses in the liver, spleen, and lymph nodes, and other cells of the reticulo-endothelial system. The most important, wandering cells are the large, mononuclear elements known as the macrophages.

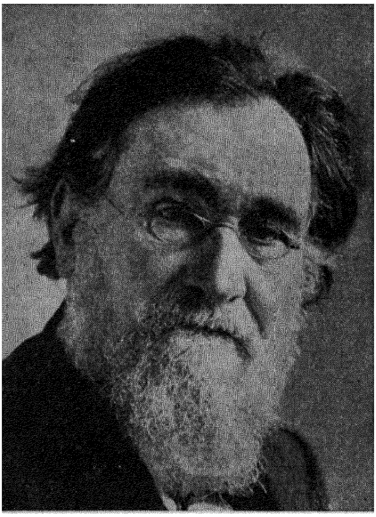


FIG. 196.—Elie Metchnikoff.

It has been shown that when leucocytes are washed free of serum and then mixed with bacteria, no phagocytosis occurs. If, however, a small amount of normal serum is added to the mixture, active phagocytosis is restored. Antibodies known as bacteriotropins, or opsonins, are present in serums that prepare the bacteria for phagocytosis. The opsonins are antibodies in the true sense because they are increased during the process of immunization and are specific in their action. They are present in normal serum and increased by immunization.

BACTERIOPHAGE (TWORT-D'HERELLE PHENOMENON)

Twort (1915) noticed certain transparent areas in a culture of *staphylococcus* that were free from bacterial growth. He found that if he touched one of these areas with an inoculating loop and then streaked it over the surface of an agar culture of the same species of *Staphylococcus*, clear transparent areas developed along the line of streaking. Twort also found that if the material from transparent areas was filtered through a Berkefeld filter, the filtrate contained a substance that was capable of dissolving a broth culture of the organisms. This lytic action was shown to be transmissible in series. D'Herelle (1916) independently of Twort observed the same phenomenon and named the lytic principle bacteriophage. The term bacteriophage, sometimes referred to simply as phage, means literally "bacteria-eating agent."

A number of bacteriophages, especially those active for the intestinal bacteria, have been obtained from feces, sewage, ground-up houseflies, etc. Extracts of chicken feces and horse manure have yielded phages

specific for dysentery and typhoid organisms. Phages have also been isolated from old laboratory cultures, and many stock cultures in all probability contain a phage of some kind. Some strains of a bacterial species may carry a phage that will not cause a lysis of the organisms. Separation of the lytic principle by filtration gives a solution that is capable of dissolving susceptible strains of the same species.

Many different phages have been isolated and studied. Bacteriophages are more or less specific in that they affect a single species or

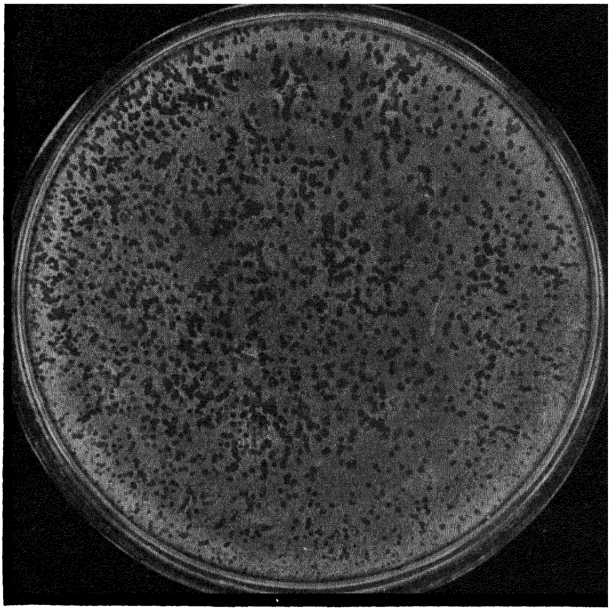


FIG. 197.—Effect of bacteriophage. Agar mixed with a suspension of *Staphylococcus aureus* + specific bacteriophage, and incubated. The clear areas represent places where growth has been eaten away by the bacteriophage.

closely related types. Organisms having similar immunological characteristics are usually lysed by the same phage.

The nature of bacteriophage is not definitely known. One group, led by D'Herelle, believes that bacteriophage is an autonomous ultramicroscopic parasite, which possesses the power to break through the outer membrane and invade the bacterial cell. The bacteriophage multiplies inside of the cell and its accumulation results in cellular destruction or lysis. The newer concept, and the view held by most investigators, is that phage is not an autonomous living agent but a protein possessing many of the properties of an enzyme.

Northrop (1938) has supplied probably the most direct proof to this concept by isolating from a lysed culture of *Staphylococcus* a nucleo-

protein which possessed the activity of the original crude preparation. Molecular weight determinations suggested that this protein existed in various sized molecules of from 500,000 to 300,000,000. The proportion of the small molecules increased as the concentration decreased. In another report Northrop (1937) found that 1×10^{-12} mg. of this nucleoprotein was capable of producing lysis when added to cultures of the specific organism. These observations are in support of the nonliving nature of bacteriophage.

When bacteriophages were first discovered it was thought that they would be of great value therapeutically. Since phage lyses organisms readily in vitro, it was believed that they would be capable of doing the same in vivo. A few years ago it was a common practice for physicians to prescribe phages for many types of infections. Unfortunately, clinical observations have not borne out this claim and it is probable that phages have no appreciable effect upon the outcome of infections. Their use for this purpose has been largely discontinued.

For further reading on infection and immunity consult Burdon (1939), Gay (1935), Krueger (1936), Krueger and Scribner (1939), Topley (1933), Topley and Wilson (1937), Wells (1929), Zinsser and Bayne-Jones (1939), and Zinsser, Enders, and Fothergill (1939).

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CHAPTER XXV

BACTERIAL AND VIRUS DISEASES OF PLANTS

BACTERIA

The first recorded observations on a bacterial disease of plants dates back to the work of Burrill (1881) who discovered the causative organism of pear blight. This work was confirmed by Waite (1891) who isolated the etiological agent and proved its pathogenicity. Since then about 200 species of bacteria have been found to be pathogenic for plants. It is safe to assume that there are as many bacterial diseases of plants as of man.

Before an organism can be stated definitely to be the causative agent of a plant disease, it must be isolated from the plant tissue and its pathogenicity proved beyond doubt. Koch (1883) postulated certain requirements that should be met before an organism can be said to be the cause of a specific disease. These requirements have been generally accepted by both plant and animal pathologists. Koch's postulates are as follows:

1. An organism must be consistently associated with the disease in question.
2. The organism must be isolated in pure culture and accurately described.
3. The organism in pure culture, when inoculated into healthy plants, must be capable of reproducing the disease.
4. The organism must be reisolated from the diseased plant tissue and shown to be identical with the original species.

The bacterial diseases of plants may be placed in five groups on the basis of the location and character of the lesions produced: (1) soft rots, (2) vascular diseases or wilts, (3) blights, (4) intumescence diseases, and (5) local lesions or spots.

SOFT ROTS

Organisms responsible for soft rots reduce the plant tissue to a soft, very moist, pulpy mass. The condition may be better recognized as a state of rottenness. The bacterial attack may or may not be due to a specific organism.

The organisms producing soft rots differ from the other forms found in the soil in that they have the power to attack healthy plant tissue by the secretion of an extracellular enzyme. The enzyme dissolves the pectin or cement-like material that binds together the plant cells. The action is probably hydrolytic, resulting in the liberation of soluble sugars

which are utilized by the bacteria for food. The result is that the plant is reduced to a mass of separate cells, which become converted later into a slimy, pulpy material.

In most cases the specific organism is accompanied or closely followed by many saprophytic soil bacteria and fungi. These organisms find a favorable environment in the exposed cells and produce relatively large quantities of ammonia by the deaminization of the amino acids present in the proteins of dead plant tissue. The ammonia produces a destruction of the neighboring plant cells and rapidly reduces the plant to a slimy, pulpy, foul-smelling mass. The unpleasant odor is due to the secondary invaders. Plants decayed by pure cultures of the specific disease organisms do not produce an objectionable odor.

The most important organisms causing soft rots include (1) *Erwinia carotovora*, which produces a rapid soft rot of roots, rhizomes, fruits, and the fleshy stems of a variety of plants, including carrot, cabbage, celery, cucumber, eggplant, iris, muskmelon, hyacinth, onion, parsnip, pepper, potato, radish, tomato, turnip, and other plants. (2) *E. phytophthora*, which produces a stem rot (blackleg) of potato. It affects also cucumbers and other vegetables. (3) *E. solanisapra*, the causative organism of soft rot of potato and other vegetables. Positive infections have been produced by inoculating the organism into many vegetables, including cucumber, carrot, radish, parsnip, cauliflower, cabbage, celery, turnip, tomatoes, etc. The organism secretes a powerful cytolytic enzyme, which dissolves the middle lamellar layer. (4) *E. flavida*, the cause of soft rot of sugar cane. Some consider the organism to be a chromogenic strain of *E. carotovora*. (5) *E. aroideae*, which produces a soft rot of calla lily. The organism also affects raw potato, eggplant, cauliflower, radish, cucumber, cabbage, parsnips, turnips, tomato, etc. Some consider this organism to be identical with *E. carotovora* but they are distinct serologically. (6) *Phytomonas campestris*, the cause of soft rot of crucifers. The organism causes a vascular infection of cabbage, cauliflower, and rutabagas. The organism is pathogenic on other vegetables.

Soft Rot of Carrot.—Jones (1901) first described this disease of carrots. It is sometimes spoken of as Jones' soft rot of carrot. The organism seldom attacks well-developed green parts. The carrots must contain considerable moisture for vigorous attack to take place.

The organism gains entrance to the plant through a wound. Multiplication takes place at a rapid rate, owing to the poisonous action of the bacterial waste products. This results in a disintegration of the susceptible parts into a soft, wet pulp. The organisms advance into the weakened tissues by way of the intercellular spaces, loosening the cells by the hydrolysis of the cement-like material or pectin that binds them together. This results in the death of the protoplasm of the separated

cells. The specific organism remains extracellular, except in the late stages of the disease.

The first signs of the disease are the appearance of water-soaked areas around the point of entry. These areas or spots may be seen in from 18 to 36 hr. after inoculation. Within 48 to 72 hr. the tissue becomes flabby and exudes a gray fluid swarming with bacteria. Under some conditions a film of bacterial growth may be seen covering the entire surface of the carrot.

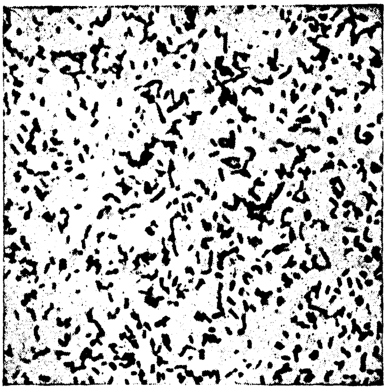

FIG. 198.—*Erwinia carotovora*, the cause of soft rot in carrots and other plants.

Carrot rot usually begins at the crown or at the root tip. The core rots more rapidly than the outer part of the root and turgid roots are more susceptible than flabby or dried ones.

Jones' disease is caused by *Erwinia carotovora*, a small grayish-white, Gram-negative, nonspore-forming, motile rod (Fig. 198). Its optimum growth temperature is 25 to 30°C. The organism is not specific for carrots alone but is able to attack a large number of plant species (see page 547).

Stem Rot or Blackleg of Potato.—The first visible signs of the disease are sudden wilting or slow yellowing of the lower leaves. The base of the shoots are blackened and softened at the surface of the earth or just below it. At first this blackening and softening are restricted to the base of the stem but soon spread to the upper parts. The stems wilt, shrivel, and fall to the ground. The tubers often decay.

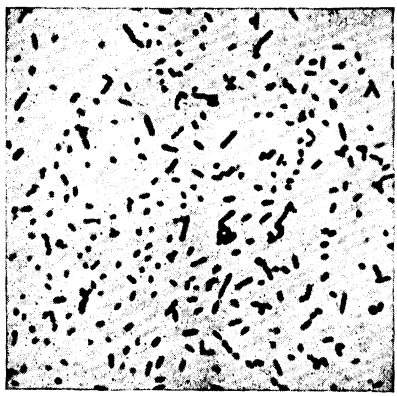

FIG. 199.—*Erwinia phytophthora*, the cause of stem rot in potato, cucumber, and other vegetables.

Inoculation of young shoots of healthy plants with a culture of the organisms reproduces the disease. Young shoots are more susceptible than old ones. The disease is more prevalent during warm moist summers and autumns and may continue through the winter months if the temperature is sufficiently high.

The organism responsible for black rot of potato is known as *Erwinia phytophthora* (Fig. 199). It is a small, white, Gram-negative, nonspore-forming, motile rod. *E. phytophthora* and *E. carotovora* are similar

morphologically and physiologically but differ serologically. Another point of difference is that *E. phytophthora* produces an inky black coloration of raw potato whereas *E. carotovora* produces very little, if any, coloration.

Potatoes grown in moist soil often become infected by entrance of the etiological agent through the lenticels. This may be easily demonstrated in the laboratory by immersing clean, sound tubers for 30 hr. in a very dilute suspension of *E. phytophthora* in distilled water. Numerous infections, centering in the lenticels, should make their appearance after several days, followed by a disintegration of the interior of the potato tubers.

Soft Rot of Crucifers.—The disease produces a soft rot of cabbage, cauliflower, and related species. Pammel (1895) first isolated the organism from diseased rutabagas.

In cabbage the organism produces a disease known as "stump rot." The decay originates in the stem and spreads into the base and head. The first symptoms usually consist of a wilting of the outer leaves followed by a cessation of growth. The head may be easily separated from the stem, leaving a rotted stump.

The disease is caused by *Phytomonas campestris*, a pale-yellow, motile, capsulated, nonspore-forming, Gram-negative rod. The optimum temperature is 28 to 30°C.

VASCULAR DISEASES OR WILTS

Some organisms multiply and accumulate in large numbers in the vascular system, causing an interruption in the flow of sap in the plant. Infections of this type are grouped under the vascular diseases. A complete interruption in the flow of sap results in a rapid wilting of the plant. A partial interruption results in the growth of a sickly plant, which makes poor headway and finally succumbs. In many cases death is due to the action of secondary invaders.

The important [illegible] causing vascular diseases in plants include: (1) *Erwinia tracheiphila*, the causative agent of cucurbit wilt. The organism also affects cantaloupes, muskmelons, pumpkins, and squashes. (2) *Phytomonas stewartii*, a vascular pathogen producing a wilt of corn. (3) *P. solanacearum*, the cause of brown rot of solanaceous plants. The organism attacks numerous species, especially potato, tobacco, and tomato. (4) *P. michiganensis*, the causative organism of canker of tomato. (5) *P. flaccumfaciens*, the cause of wilt disease of beans.

Cucurbit Wilt.—Several plants are susceptible to an infection characterized by wilting of the vines, followed by shriveling and death. It is a widespread, typically vascular disease, transmitted by the bite of

insects. The causative agent attacks cucumbers, cantaloupes, muskmelons, pumpkins, and squashes.

First signs of the disease make their appearance on the leaves in the form of dull-green, flabby patches. Later the leaves wilt and shrivel. The organisms pass downward into the stem by way of the spiral vessels into the petiole. The bacteria rupture the vessel walls and invade the intercellular spaces of the parenchyma. The cut surfaces of diseased stems exude a white, viscid liquid heavily infected with bacteria. The bacteria secrete a cytolytic enzyme, which dissolves the middle lamellar layer of the parenchyma cells.

The disease is caused by a small, grayish-white, viscid, nonsporeforming, motile rod known as *Erwinia tracheiphila*. The organism is capsulated and Gram-negative. It grows very slowly even at 30°C., its optimum temperature.

If a leaf is pricked with a needle, previously dipped in a culture of the organisms, the rods make their way down the petiole of the leaf into the stem. The organisms multiply and accumulate in large numbers in the water ducts. The incubation period is about one week, with symptoms appearing first on the leaf.

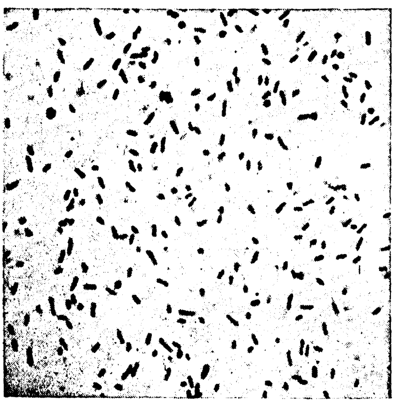

FIG. 200.—*Phytomonas stewartii*, the cause of blight of sweet corn.

Bacterial Wilt of Corn.—This is sometimes referred to as Stewart's disease of corn. The causative agent is confined principally to corn, especially to sweet corn. The disease is characterized by a shriveling of the foliage. When the stem of an infected plant is cut, a yellow slime oozes from the vascular bundles. Infection generally occurs during the seedling stage through the stomata. The organisms occur in great numbers in the vessels and tend to come to the surface of the husks through stomata. The kernels become heavily infected with the organisms. Some plants are destroyed during the seedling stage; others reach maturity before showing secondary signs of the disease. This is an excellent example of a seed-borne infection.

The organism responsible for the disease is known as *Phytomonas stewartii*, a small, nonmotile, capsulated, Gram-negative rod (Fig. 200). It produces a yellow pigment. The organism grows best at a temperature of 30°C.

Brown Rot of Solanaceae.—The first sign of the disease is a sudden wilting of the leaves on a branch or on the entire plant. The stems

shrivel and eventually die. The bacteria are found first in the vascular bundles and then enter the parenchyma cells of the cortex and pith. The vascular bundles are usually stained brown or black. Sometimes an alkaline slime oozes to the surface but often the surface is sound. The bacteria spread through the vascular bundles of the stolons and eventually reach the tubers. The vascular ring is first destroyed, followed by decay of the storage tissues. If a diseased stem or tuber is cut, the bacteria ooze out in a brownish slime.

The organism responsible for the disease is known as *Phytomonas solanacearum.* It is a small, motile, Gram-negative rod, producing a brownish-colored pigment on media. The organism attacks numerous plants including tomato, tobacco, and potato.

Bacterial Canker of Tomato.—This disease was first observed in Grand Rapids, Mich., and for some years was known as the Grand Rapids disease. It is an infectious, parenchymo-vascular wilt of tomato. It is quite similar to the brown rot disease of Solanaceae and is often confused with it.

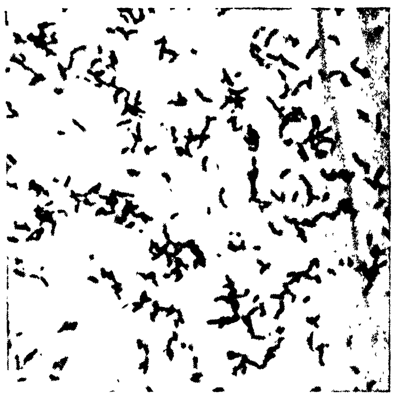

FIG. 201.—*Phytomonas michiganensis,* the cause of canker in tomato.

The leaves are slowly attacked and shrivel one after another. The meristem is attacked and destroyed, resulting in the formation of cavities. The organisms show a strong tendency to come to the surface of infected leaves, fruits, and shoots affording an excellent opportunity for the spread of the infection to neighboring plants. The bacteria usually enter new plants through the stomata. The disease is primarily an infection of the phloem.

The causative agent of the disease is *Phytomonas michiganensis,* a small, nonmotile, capsulated rod (Fig. 201). The organism is Gram-positive. Colonies on agar possess a mustard-yellow color.

BLIGHTS

Organisms producing blight diseases are capable of penetrating considerable distances between the cells, leaving the neighboring tissue intact. The bacteria grow in the plant juices without producing any digestion of the tissues. The rods usually produce a discoloration of the leaves and branches. Death is probably due to an interference with the flow of the plant sap.

A partial list of the organisms producing blights includes: (1) *Erwinia* . . the causative agent of fire blight or pear blight. The organism

attacks a large number of species of the family Rosaceae. (2) *Phytomonas phaseoli*, the cause of bean blight. The organism attacks the hyacinth bean, the lupine, and other plants. (3) *P. mori*, the etiological agent of mulberry blight. (4) *P. medicaginis*, the cause of stem blight of alfalfa. (5) *P. pisi*, the etiological agent of stem blight of field and garden peas. (6) *P. juglandis*, the causative organism of walnut blight. (7) *E. lathyri*, the organism responsible for streak disease of sweet peas and clovers.

Fire Blight.—The organism attacks the blossom clusters and tips of growing twigs. The leaves attached to the affected parts usually turn brown or black, the petioles blacken, and the bark of young twigs becomes darkened and shriveled. Since the twigs have the appearance of green brush that has been partly burned, the disease is sometimes known as fire blight. The blighted leaves cling tenaciously to the diseased twigs, even after the unaffected leaves have fallen in the autumn, thus affording an easy method for the recognition of the disease.

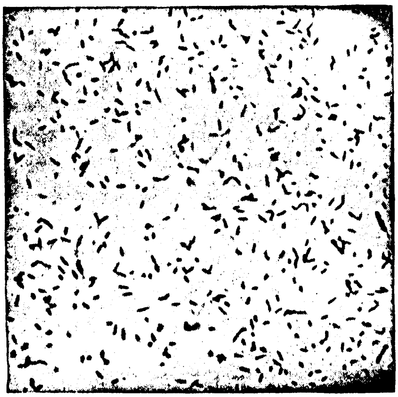

FIG. 202.—*Erwinia amylovora*, the cause of blight on pear and apple trees.

Sometimes the disease spreads to the larger limbs and trunk of the tree where it produces body blight. The bark becomes cracked and a thick sticky liquid oozes from the injured surface. The bark continues to split as the disease progresses and the injured area becomes rough and develops a canker.

The unripe fruit becomes blackened, shriveled, and hardened. A sticky exudate usually oozes from the fruit and appears on the surface. Microscopic examination of the sticky liquid shows the presence of dense masses of motile organisms.

The disease is caused by *Erwinia amylovora*, a small, motile, Gram-negative rod (Fig. 202). The organism attacks pear, apple, and many other members of the family Rosaceae.

Bean Blight.—The organism causing bean blight attacks the parenchyma and vessels of leaves, stems, and pods. The disease is characterized by the appearance of minute translucent dots on the leaves, which gradually coalesce, enlarge, and usually retain a water-soaked border. The spots finally become sunken and discolored. Irregular reddish, yellowish, or brownish spots are formed. The uninfected parts of the green leaf frequently persist around the discolored, infected areas.

Small, circular spots surrounding a stoma appear on the pods. The spots slowly enlarge and finally become sunken and discolored. Bacteria may be forced through the stomata in large numbers. The organisms multiply at a faster rate on the pods than on the leaves. In very severe cases the leaves shrivel and finally fall off. The pods become spotted, dwarfed, and worthless. In damp weather both leaves and pods may become moldy.

The disease is caused by *Phytomonas phaseoli*, a small, motile, yellow-pigmented, nonsporulating, Gram-negative rod. The organism is also pathogenic for the hyacinth bean, the lupine, and other plants. It does not attack the soybean.

Mulberry Blight.—In many respects this disease resembles fire blight of the pear. The young shoots show dark, sunken, longitudinal stripes. A whitish or yellowish liquid often oozes into the sunken areas, which dries to a glossy finish. If the surface is dry, the liquid may exude from the lenticels. Both the wood and the bark are invaded in the young shoots and the end of the disease is either a shriveling of the affected parts followed by death, or a curved, one-sided growth. On the older parts of the tree the disease is present in the form of cankerous patches. The bacteria show a strong tendency to ooze to the surface of the diseased parts.

The leaves show the presence of many spots, which tend to coalesce and enlarge. The spots are first water-soaked in appearance, then become brown or black. The surrounding tissues become yellow in color. The bacteria travel in the intercellular spaces and produce cavities. Dark sunken spots appear on the veins and midrib of the leaves. Leaves attacked early show considerable distortion in shape. The disease is seldom fatal but the trees often become dwarfed and ragged in appearance.

The disease is caused by *Phytomonas mori*, a motile, Gram-negative rod. The organism appears to attack only various kinds of mulberry trees.

Stem Blight of Alfalfa.—This disease is primarily a stem infection. The stems first show a watery, semitransparent, yellowish or greenish color along one side. This is followed by the appearance of a thick, clear, viscid liquid, which spreads over the surface of the diseased area and tends to collect in bead-like droplets. The exudate dries to a smooth, glassy finish giving the stems a varnished appearance. The stems become quite brittle and easily broken. The leaves attached to the blighted stems usually become infected. Sometimes the leaves become infected and the stems remain normal. If this occurs, the petioles become water-soaked, turn pale yellow, and then droop. The infection may remain confined to the petiole and base of the leaflet or it may involve the entire blade.

Plants one year old may show blackened areas in the crown and black streaks extending to the taproot. The blackening increases in other plants until the whole crown becomes infected. The crown buds are either destroyed or the root is no longer capable of performing its functions, and the plant dies.

Slides prepared from small pieces of the watery tissue or from a fragment of the dried exudate reveal masses of motile, rod-shaped organisms. The causative agent of the disease is known as *Phytomonas medicaginis*, a small, Gram-negative, rod-shaped organism. The organism apparently attacks only the alfalfa plant.

Stem Blight of Field and Garden Peas.—The infection occurs in both field and garden peas and the symptoms are similar to those of stem

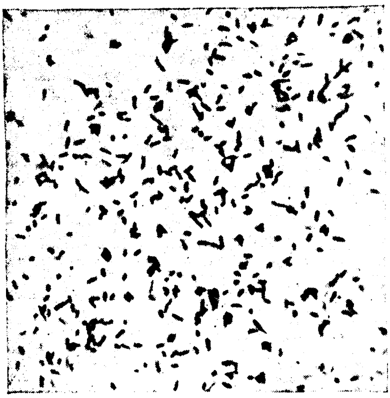

FIG. 203.—*Phytomonas pisi*, the causative agent of stem blight in field and garden peas.

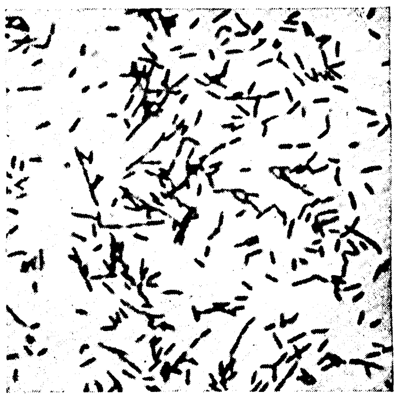

FIG. 204.—*Phytomonas juglandis*, pathogenic for the English walnut.

blight of alfalfa. The stems have a watery appearance and become first green, then olive brown, and finally dark brown in color. The leaves are first watery, then turn yellow in color. This may be followed by wilting. Discoloration of the stems of young plants is followed by shriveling and death. Older plants may follow a similar cycle, although the disease usually runs a milder course. In some cases the older plants outgrow the blight.

The infection is caused by *Phytomonas pisi*, a small, motile, Gram-negative rod (Fig. 203). The organism produces a greenish fluorescent pigment.

Walnut Blight.—The disease affects all the new, tender, growing parts of the tree, such as the petioles of the leaves, veins, and adjoining parenchyma, young nuts, and branches. The disease starts in the new growth on the branches and produces small, discolored areas, which may extend for a distance of 2 to 3 in. along the green shoot. The central

portion of the lesion becomes black and the margin becomes water-soaked. Later the entire diseased area turns black and possesses a shrunken, dried-out, deformed, and cracked appearance. If the symptoms are mild, only the bark and wood are diseased; if the symptoms are severe, the tissue is destroyed as far as the pith. The infection is checked by a hardening of the wood. This does not affect the vitality of the tree to any extent. Sometimes the petioles and veins of the leaves become blackened and browned while the intermediate tissue may show brown, circular, or angular spots. The nuts become heavily infected with the organisms and drop from the tree when they are very small. Badly diseased groves may show a loss of 50 per cent of the crop. The infection usually starts at the blossom end. The lesions first appear as small, circular, raised, discolored, water-soaked areas. These gradually increase in size and become black. The disease may extend through the hull and shell into the kernel, which becomes blackened and destroyed.

The causative agent is *Phytomonas juglandis*, a small, motile, Gram-negative rod (Fig. 204). Colonies on agar have a pale-yellow color.

INTUMESCENCE DISEASES

Some bacteria have the power to produce galls or tumors on plants. These excrescences or abnormal growths are due to the action of organisms on the meristematic tissue of the plants. Tissues infected in this manner are grouped under the intumescence diseases.

In some infections the galls remain small; in others they may assume large proportions. Sugar beets have been known to carry tumors larger than the original plant. Some believe that tumor infections in plants are similar to cancerous growth in animals. The bacteria produce an irritation of the plant cells resulting in rapid division. Intracellular organisms are not necessary for the development of the characteristic lesions. Rapid cell division is probably the result of the action of some irritating product produced by the growth of the bacterial organisms.

The important organisms producing intumescence diseases include (1) *Phytomonas savastanoi*, the cause of olive knot; (2) *P. tumefaciens*, the causative agent of crown galls on Paris daisy; cross-inoculable on many other plants; (3) *P. tonelliana*, the etiological agent of galls on the oleander plant; (4) *P. pseudotsugae*, the cause of galls on the Douglas fir in California; and (5) *P. gypsophilae*, the organism responsible for the development of galls on *Gypsophila paniculata* and related plants.

Olive Knot.—The causative agent of this disease is responsible for the development of tumors or excrescences on olive trees. The attacked parts are dwarfed or killed, and occasionally the entire tree is destroyed. Usually the tree is stunted or prevented from bearing fruit. New tumors or galls often develop around dead knots and also at a distance

from the original lesion. The entire tree is susceptible to attack by the specific organism. A tree seldom recovers from an infection. The organisms continue to invade new shoots and also new parts of the old tree. The organisms may be readily seen between the cells and in intercellular cavities. The surrounding tissue has a water-soaked or brownish appearance. During wet or rainy weather the organisms are secreted from the tumors from which they are washed to other parts of the same tree and probably to other trees. Tree wounds offer a portal of entry to the organisms, resulting in new infections. The galls may contain both wood and bark. Often a large part of the tumor is composed of bark, giving rise to a soft, cheesy gall.

The disease is caused by *Phytomonas savastanoi*, a small, white, motile, Gram-negative, nonspore-forming rod. The optimum temperature of the organism is 23 to 24°C. It produces a soluble brown pigment on potato.

Crown Gall.—This is another gall or tumor disease of bacterial origin. The causative agent is cross-inoculable on a large number of plants, including chestnut, sugar beet, willow, wild fig, hop, grape, clover, almond, raspberry, apple, Paris daisy, Japanese radish, cauliflower, tobacco, castor, carnation, orange, mango, and sunflower.

The organism attacks primarily the parenchyma. The cells are not killed but are induced to multiply at a more rapid rate. This results in the development of an imperfectly vascularized, covered or naked, irregular excrescence or tumor. The overgrowth is composed, in part at least, of masses of rapidly multiplying, round, or spindle-shaped cells of reduced size. Under some conditions and on certain plants the overgrowth may be larger than the root or stem that bears it, but it frequently decays readily.

The disease possesses the following characteristics:

1. The growth is extraphysiological and produces an injurious action on the whole plant. The result is that the plant is slowly dwarfed and finally killed.

2. Secondary overgrowths occur from the primary tumor. The new growths are derived by cell division, in the form of a continuous chain of cells, from the primary tumor.

3. The new tumors reproduce the structure of the tissues in which the initial overgrowth has developed. This means that if the primary growth is in the stem and the secondary tumor is in the leaf, the attacked leaf will be converted into a stem-like organ.

The plant is seldom destroyed but the attacked branches are usually killed. The infected parts are generally dwarfed in their development. If the tumor is centrally located, the plant may be easily destroyed. The organisms are present in only some of the new cells. Bacteria

are carried over into the daughter cells, but multiplication does not take place in all the new cells. The organisms are not present in large numbers and, owing to the granular nature of the protoplasm, are seen with great difficulty even with the oil-immersion objective.

The organism responsible for crown gall of plants is called *Phytomonas tumefaciens* (Fig. 205). It is a small, white, nonspore-forming, motile, Gram-negative rod. The organism is very pleomorphic in the presence of such compounds as acids and sodium chloride. These abnormal forms are frequently present in tumors. The optimum growth temperature is 25 to 28°C.

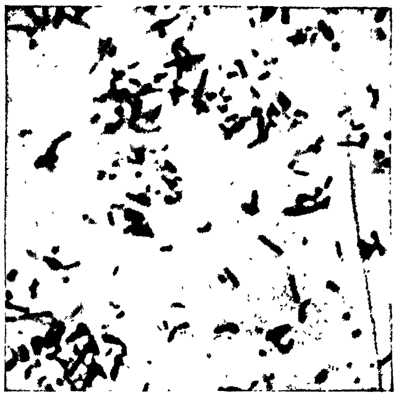

FIG. 205.—*Phytomonas tumefaciens*, the cause of crown gall of plants.

LOCAL LESIONS OR SPOTS

In many diseases of plants the attack is restricted to a small area around the point of entry. These diseases are grouped under local lesions or spots. The organisms responsible for leaf-spot diseases produce a vigorous attack on the plant tissue with the result that the cells become heavily infected and strongly discolored. The discolored areas dry up and frequently fall out, leaving holes in the leaves.

Some of the organisms producing spot diseases are: (1) *Phytomonas cucurbitae*, the cause of leaf spot of squash and related plants. (2) *P. malvacearum*, the agent responsible for angular leaf spot of cotton. It causes a leaf spot, a stem lesion, and a boll lesion. (3) *P. ricinicola*, the causative agent of leaf spot of castor bean. (4) *P. vesicatoria*, the cause of spotted tomato fruits in South America. (5) *P. begoniae*, the cause of leaf spot of begonia. (6) *P. angulata*, the organism responsible for angular leaf spot of tobacco. (7) *P. maculicola*, the agent responsible for cauliflower spot. (8) *P. mellea*, the cause of brown rusty spots on Wisconsin tobacco.

Cauliflower Spot.—The organism causing this disease attacks both cauliflower and cabbage. The disease is characterized by a spotting of the leaves on the veins and in the parenchyma. The spots are first water-soaked, then become brownish to purplish gray by reflected light, and thin and almost colorless by transmitted light. The spots are usually small, ranging from 1 to 3 mm. in diameter. Larger spots may be formed by coalescence of two or more small ones. Badly spotted leaves may turn yellow, then finally drop off of the plant. The infection takes place apparently through the stomata because each spot has a

stoma in its center. Infection can be transmitted to healthy plants by spraying the leaves with a culture of the organism. Old and very young leaves appear to be partly or entirely immune to infection.

The disease is caused by *Phytomonas maculicola*, a small, white, motile, nonsporulating, Gram-negative rod. Its optimum temperature is 24 to 25°C. The organism loses its virulence readily on laboratory media.

Angular Leaf Spot of Tobacco.—Tobacco leaves become injured by the tobacco worm crawling over the surface. The prolegs of the worm are equipped with hooks for clinging to and moving over the surface of the tobacco leaf. The hooks, in clinging to the leaf, make small punctures, which serve as points for the bacteria to enter. It is quite likely that the legs of the worm are contaminated with the specific organism of the disease. The organisms produce small angular spots on the tobacco leaf.

The organism responsible for the disease is known as *Phytomonas angulata*, a small, motile, nonspore-forming, Gram-negative rod. Colonies on agar are white in color.

MODE OF INFECTION

The mode of entry of bacteria into the plant is usually through wounds. Roots, leaves, and stems are easily injured mechanically by means of agricultural implements, by animals, etc. Plants become easily infected following injury to the roots, whereas sound plants remain free from bacterial attack. Hailstones are known to produce injury to plants and make them vulnerable to infection. However, the usual cause of plant injury is through the bite of various insects. Sometimes the insects carry the etiological agent on their mouth parts, making it possible to injure and infect the plant in one operation.

In many of the leaf and fruit infections the organisms gain entry through natural openings known as stomata. The organisms pass from the stomata into the intercellular spaces. The bacteria greatly reduce the resistance of the cells, by suffocation or poisoning, and make it possible for the etiological agent to enter the affected plant cells.

Bacteria may enter plants by way of the hydathodes or organs for the excretion of water. An excessive elimination of water results in the collection of considerable moisture on the plant surface. Bacteria readily collect in the water droplets making it possible for some to gain entrance to the plant.

Lenticels are also unprotected openings, which may offer bacteria a path for invasion of the plant. These organs are cortical pores in the stems of woody plants through which air penetrates to the interior.

Many insects are responsible for plant infections. Their proboscides or legs act as carriers of bacteria that are capable of attacking the plant. This is especially true of those plants which produce nectars designed to attract bees and other insects for the fertilization of flowers.

For additional reading on bacterial diseases of plants consult the articles and monographs by Bergey, Breed, Murray, and Hitchens (1939), Bonde (1939), Elliott (1930), Fawcett (1936), Heald (1933, 1937), Leach (1940), Melhus and Kent (1939), Owens (1928), Paine (1929), Smith (1920), Stevens and Hall (1933), Walker (1939), and Wormald (1939).

VIRUSES

There are probably more plant diseases caused by viruses than by bacteria. They are the cause of some of the most destructive diseases of agricultural crops. It is safe to say that almost all cultivated plants are affected by at least one virus. It is not uncommon to encounter plants affected by two or more viruses. For example, the potato is susceptible to at least 25 viruses and the tobacco to at least 12 virus infections. Because of these multiple infections it is often very difficult, if not impossible, to identify a virus by the symptoms produced.

It is a difficult matter to identify viruses because their true nature is not yet known. It was formerly believed that viruses were ultramicroscopic organisms parasitic on plants, but work of recent years appears to point to the fact that they may be inanimate and consist of proteins of high molecular weight. The presence of this protein in living cells stimulates the production of more protein of the same kind. Until the true nature of viruses is known their identification will continue to be uncertain.

AGENTS RESPONSIBLE FOR THE DISSEMINATION OF VIRUSES

Probably all virus diseases are disseminated by insects. Other methods are also capable of transferring viruses to plants but these are of secondary importance. Chief among these may be mentioned: (1) wind, (2) water, (3) soil, (4) seed, and (5) pollen.

Wind.—Wind plays a minor role in the dissemination of plant viruses. However, there are a few exceptions. The tobacco mosaic virus, for example, is very resistant to desiccation and may be spread by wind in the form of dried, crumbled plant tissue. The virus is capable of readily infecting healthy plants through slight wounds.

Water.—Water appears to be a minor factor in the spread of the great majority of plant virus diseases. Here again there are a few notable exceptions. It is possible to infect healthy plants with tobacco

necrosis by bathing the roots in water containing the virus. The virus can infect healthy plants without the aid of artificial wounding.

Soil.—The soil itself is not an agent for the transmission of virus diseases. Infec[illegible]y occur through roots and other underground parts of plan[illegible]y [illegible] w[illegible]er, insects working in the soil, etc. Since it is very difficult to observe underground parts of plants, the mechanism for infection by this route remains obscure. However, it has been definitely shown that wheat mosaic virus may be transmitted underground through roots, or the crown, or both. The virus is capable of surviving in the soil for some time and is difficult to remove from soil by thorough washing. The mechanism for the entrance of the virus into the plant is not known.

Seed.—Seed transmission of plant diseases does occur for some viruses. Bean mosaic virus disease occurs by this route to the extent of about 50 per cent of the plants under experimental conditions. The results are usually inconsistent. A plant may show both healthy and infected seeds. Plants of the family Leguminosae appear to be more susceptible to infection by this route than plants of other families.

Pollen.—It has been shown that virus infections may be transmitted to seeds by pollen from infected plants. In the plant *Datura stramonium*, or Jimson weed, up to 79 per cent of the seeds may become infected. Bean seeds may also be infected in this manner.

Insect Transmission.—The most important agents for disseminating plant viruses are insects. Many of them are known to transmit virus diseases. Some insects transmit the virus mechanically; others transmit the infection biologically. The former method usually occurs in those insects which have chewing mouth parts. The latter method occurs only in the sucking insects but transmission by these insects is not always biological.

According to Leach (1940) biological transmission of plant viruses by insects usually has one or more of the following attributes:

1. An apparent multiplication or increase of the virus in the insect's body.
2. An incubation period in the body of the insect, *i.e.*, a necessary period after feeding on infected plants before the insect becomes infective or viruliferous.
3. A degree of specificity between the insect and the virus that it transmits.
4. An obligatory relationship.
5. A relation between the age or life stage of the insect and its ability to transmit the virus.
6. Congenital transmission of the virus from one generation to the next.

For additional reading on virus diseases of plants consult the articles and monographs by Bawden (1939), Bawden and Pirie (1937), Gortner (1938), Grainger (1934), Heald (1933, 1937), Holmes (1939), Leach (1940),

Rawlins and Takahashi (1938), Smith (1934, 1937), Stanley (1935, 1936*a*,*b*, 1938), and Storey (1939).

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CHAPTER XXVI

SPECIFIC INFECTIONS

Bacteria.—Thousands of species of bacteria have been isolated and studied but only a very small number of these are capable of producing infections in man and lower animals. The important types of disease-producing bacteria capable of infecting man probably do not greatly exceed 100 species. Some organisms are quite specific in that they attack only one host; others are less specific, being capable of naturally infecting more than one host. Measles and scarlet fever occur naturally only in man. The organism that produces glanders in horses attacks also goats, sheep, dogs, cats, rabbits, guinea pigs, and man. The anthrax organism attacks not only cattle, sheep, and horses, but also man. Diseases of man and animals are caused not only by bacteria but by other classes of organisms such as protozoa, spirochaetes, molds, yeasts, and viruses.

Protozoa.—A large number of protozoa of great importance are responsible for diseases in man and animals. Among these are the organisms of malaria (*Plasmodium vivax*, *P. malariae*, and *P. falciparum*) belonging to the class Sporozóa; African sleeping sickness caused by *Trypanosoma gambiense*, and *T. rhodesiense* of the class Mastigophora; and the causative agent of amoebic dysentery (*Entamoeba histolytica*) classed under the Sarcodina.

Spirochaetes.—The spirochaetes, capable of producing infections, include some well-known representatives. The best known member of this group is *Treponema pallidum*, the causative agent of syphilis. The fusiform bacillus *Fusobacterium plauti-vincenti* and the spirochaete *Borrelia vincentii*, growing in association, produce the disease known as Vincent's angina or trench mouth.

Yeasts.—Many species of yeasts or yeast-like organisms are known that are parasitic on man and animals. An ulcerative disease of the mouth and throat known as thrush is caused by the yeast-like organism *Oidium albicans* (*Monilia albicans*). The organism shows a tendency to grow as filaments. Blastomycosis is an ulcerative infection of the skin and subcutaneous tissues. The organism sometimes invades the internal organs, such as lungs, spleen, and kidneys and the bones. The disease is caused by the yeast-like organism *Blastomyces dermatitidis* (*Oidium dermatitidis*, *Cryptococcus gilchristi*, *Mycoderma gilchristi*, *M. dermatitis*, etc.).

Molds.—The molds produce several very important infections in man. Probably the best-known member of this group is the organism responsible for the infection known as "athlete's foot" or ringworm. It is caused by several species belonging to the genus *Trichophyton.*

Coccidioidal granuloma is produced by the mold *Coccidioides immitis* (*Oidium coccidioides, O. immitis, Mycoderma immitis,* etc.). The disease manifests itself in so many forms that no general description can be given. Bronchial or pulmonary lesions are almost always present. The skin and subcutaneous tissues are usually involved. The lesions consist of firm or soft nodules, abscesses, ulcers, sinus infections, etc. The bones and joints may also be involved. The organisms appear in the tissues as large, round, or spherical cells with thick cell walls. They are sometimes spoken of as double contoured bodies. Spores appear in the larger cells. On maturity the spore-filled cells rupture and release the spores. Each spore increases to full size and then repeats the cycle.

DISEASES OF MAN AND ANIMALS

The number of pathogenic organisms is so large that it is beyond the scope of a textbook on fundamentals to give a detailed discussion of each disease and its specific etiological agent. For this reason only a brief outline of the most important diseases of man and animals is included for convenient reference. Many excellent textbooks on the disease organisms are listed at the end of this section and may be consulted by those interested in the subject.

The species classified under their respective genera are discussed under the following subheadings:

Name and characteristics of the organism:

1. Disease produced.
2. Source of infection.
3. Mode of transmission.
4. Immunity.
5. Prevention and control.

These subheadings are referred to in the text by their respective numbers, not by their exact titles. The genera are arranged alphabetically.

ACTINOBACILLUS

Actinobacillus lignieresi.—A small rod 0.4 by 1.0 to 1.5μ, nonmotile, does not produce spores, aerobic, and Gram-negative.

1. Produces actinobacillosis of cattle and swine; slightly pathogenic for rabbits and guinea pigs. Lesions found in soft tissues, usually lymph nodes, where granulomatous tumors are formed. These eventually break down to form abscesses.
2. Foods, abscesses of the skin, etc., are a source of infection.
3. Natural infection believed to occur by ingestion of foods containing the organisms.
4. No acquired immunity.
5. Disease controlled by destruction of infected animals.

ACTINOMYCES

Actinomyces bovis.—Thin, branching filaments with branching hyphae, 0.4 to 0.6μ in thickness. Large, club-shaped forms present in animal tissues. Gram-positive.

1. The cause of actinomycosis in cattle and swine, and sometimes in man (Fig. 206).
2. Foods, saliva, pus, nasal discharge.
3. Transmitted by infected pus, saliva, or nasal discharge.
4. No acquired immunity.
5. Care should be taken to prevent discharge from lesions from coming in contact with man and animals. No specific treatment known. Large doses of iodides give excellent results in cattle.

Actinomyces hominis.—Mycelium and hyphae straight. Filaments show branching. Club-shaped forms develop in tissues. **Conidia appear** in loose spirals and do not fragment readily. Organism nonspore-forming, nonmotile, nonacid-fast, aerobic, and microaerophilic. Gram-positive.

1. Produces actinomycosis in man.
2. Grains, grasses, air, saliva, pus, nasal discharge.
3. Transmitted by foods, air, pus, saliva, or nasal discharge.
4. No acquired immunity.
5. Care should be taken to prevent contact with lesions. No specific treatment known.

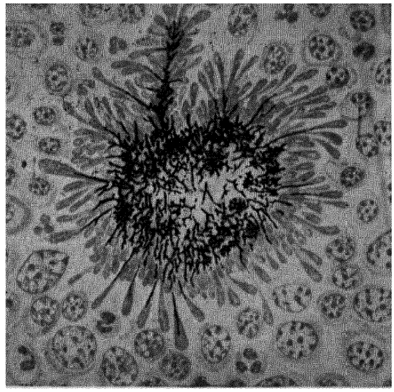


FIG. 206.—*Actinomyces bovis.* Section of an actinomycotic lesion from the jaw of an ox. The disease is sometimes referred to as "lumpy jaw" of cattle. Note the filamentous mycelium surrounded by club-shaped forms. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Actinomyces madurae.—Mycelium and hyphae straight and branched. A few open spirals occasionally formed. Filaments 1.0 to 1.5μ in thickness. The ends break up into ovoid conidia. Club-shaped forms appear in lesions. Organism aerobic, nonmotile, usually nonspore-forming, and nonacid-fast. Gram-positive.

1. The cause of madura foot, a chronic, suppurative granulomatous disease of man. Disease usually affects the foot, but other parts of the body may be involved. Nonpathogenic for animals.
2. Common in tropical countries, particularly where people walk barefooted. Organism probably present in the soil.
3. Organisms introduced into the tissues through trauma.
4. No acquired immunity.
5. Not known.

Actinomyces necrophorus.—Individual rods 0.5 to 1.5μ in width forming long filaments up to 80 to 100μ in length. Branching seen occasionally. Gram-negative.

1. Produces diphtheria in cattle; gangrenous dermatitis in horses and mules; multiple necrotic foci in liver of cattle and hogs. May be transmitted to mice and rabbits.
2. Probably saliva and nasal discharge.
3. Transmitted by infected saliva or nasal discharge.
4. Probably no acquired immunity.
5. Isolation of infected animals. No specific treatment known.

BACILLUS

Bacillus anthracis.—Rods are 1.0 to 1.25 by 5 to 10μ with square to concave ends. Occur usually in long chains. Spores produced in culture and measure 0.7 to 0.8 by 1.5 to 1.8μ, central, and naked. Organisms capsulated, nonmotile, and facultative aerobic. Gram-positive.

1. The cause of anthrax, an acute specific disease of cattle, sheep, and swine, sometimes occurring in workers handling wool and hides of animals affected with the disease. Usually occurs as a febrile disease in animals that runs a rapid course and terminates in a septicemia. Two forms occur in man, *viz.*, cutaneous (malignant pustule) and internal anthrax (Fig. 207).

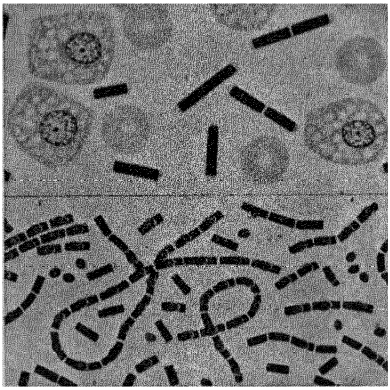


FIG. 207.—*Bacillus anthracis.* *A*, smear from the liver of an experimentally inoculated guinea pig; *B*, smear from a 72-hr. agar slant culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

2. Food, water, contact with diseased animals or their products, such as hides, wool, and hair.

3. Ingestion of food or water containing anthrax spores and through their inhalation.

4. Artificial active immunity for animals by means of vaccines.

5. Isolation of infected animals or human beings, disinfection of excreta, incineration of dead animals or deep burial in quick lime. Animals may be successfully vaccinated against the disease. Immune serums, prepared by immunizing sheep or horses against the organism, have proved of value in the treatment of human anthrax.

BORRELIA

Borrelia duttonii.—Spiral forms, 0.2 to 0.5 by 14 to 16μ. Organisms show a long, curved, delicate projection at each extremity. Anaerobic.

1. The cause of West African relapsing fever. Pathogenic for rats and mice (Fig. 208).

2. Organisms present in the blood stream of infected individuals.

3. Disease transmitted by the bite of the bloodsucking tick *Ornithodoros moubata.*

4. Immune bodies produced during the course of the disease, which are sufficiently powerful to overcome the blood infection resulting in the disappearance of the organisms from the circulation.

5. Disease easily cured in humans by the injection of salvarsan or of sodium potassium bismuth tartrate.

Borrelia recurrentis.—Organism cylindrical or slightly flattened, measuring 0.35 to 0.5 by 8 to 16μ. Ends pointed. Distance between curves is 1.5μ. Terminal spiral filament present.

1. The cause of European relapsing fever. Can be transmitted to man, monkeys, mice, and rats (Fig. 209).

2. Organisms present in the blood stream of infected individuals.

3. Disease believed to be transmitted by the common bedbug *Cimex lectularius.*

4. Immune bodies produced during the course of the disease. Infection is never fatal.

5. Destruction of bedbugs and other bloodsucking insects. Disease cured by the injection of salvarsan or of sodium potassium bismuth tartrate.

Borrelia novyi.—Spiral forms 0.3 by 17 to 20μ. Similar to *B. recurrentis* morphologically but dissimilar serologically.

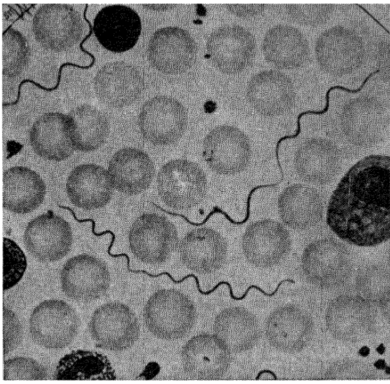


FIG. 208.—*Borrelia duttonii.* Blood smear from a case of West African relapsing fever. It is sometimes referred to as African tick fever. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

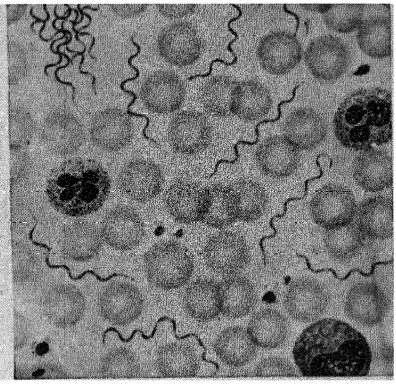


FIG. 209.—*Borrelia recurrentis.* Blood smear from a case of European relapsing fever. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

1. The cause of American relapsing fever.
2. Organisms present in the blood stream of infected individuals.
3. Disease said to be transmitted by the bite of the bloodsucking tick *Ornithodoros turicata.*
4. Immune bodies produced during the course of the disease. Infection probably never fatal.
5. Destruction of insect vector. Disease cured by injection of salvarsan or of sodium potassium bismuth tartrate.

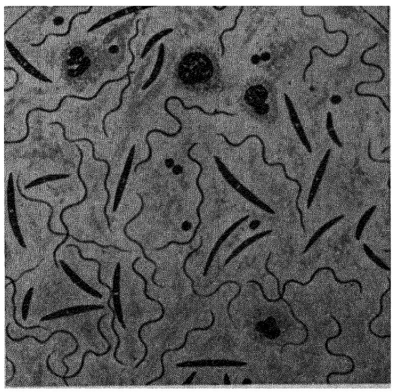


FIG. 210.—*Borrelia vincentii* and *Fusobacterium plauti-vincenti* growing in association. Smear prepared from a throat swab, taken from a case of Vincent's angina. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Borrelia kochii.—Similar to *B. duttonii* morphologically but different serologically.

1. The cause of African relapsing fever. Pathogenic for rats and mice.
2. Organisms present in the blood stream of infected individuals.
3. Disease transmitted by the bite of the tick *Ornithodoros savingnyi,* and probably other ticks.
4. Immune bodies produced during the course of the disease which are sufficiently powerful to overcome the blood infection resulting in the disappearance of the organisms from the circulation.
5. Disease cured by the injection of salvarsan or of sodium potassium bismuth tartrate.

Borrelia carteri.—Spiral forms 0.2 to 0.5 by 10 to 30μ.

1. The cause of relapsing fever in India.

2. Organisms present in the blood stream of infected individuals.

3. Disease transmitted by the bite of the Indian bedbug *Cimex rotundatus.* The disease is transmissible to monkeys, rabbits, guinea pigs, rats, and mice.

4. Immune bodies produced during the course of the disease which are sufficiently powerful to overcome the blood infection, resulting in the disappearance of the organisms from the circulation.

5. Disease cured by the injection of salvarsan or of sodium potassium bismuth tartrate.

Borrelia vincentii.—Spirals measure 0.3 by 12 to 25μ.

1. Occurs in association with *Fusobacterium plauti—vincenti* in Vincent's angina, and related infections (Fig. 210).

2. The oral cavity.

3. The disease is not ordinarily communicable. Under unusual conditions of crowding, such as may prevail among soldiers, the infection may become transmissible. The disease appears to be associated with a state of lowered resistance. The tonsillar ulceration occurs often in individuals whose resistance has been lowered by diseases such as measles, tuberculosis, diabetes, and scarlet fever.

4. No immunity.

5. The disease usually yields to local treatment. The most effective drug for this purpose appears to be salvarsan, an arsenic compound employed in the treatment of syphilis.

BRUCELLA

Brucella abortus.—Short, oval rods 0.3 to 0.4μ in length. Occur singly and in pairs, occasionally in chains. Nonmotile, nonacid-fast, and microaerophilic. Gram-negative. Organism requires 10 per cent CO_2 for isolation; becomes aerobic after several transfers.

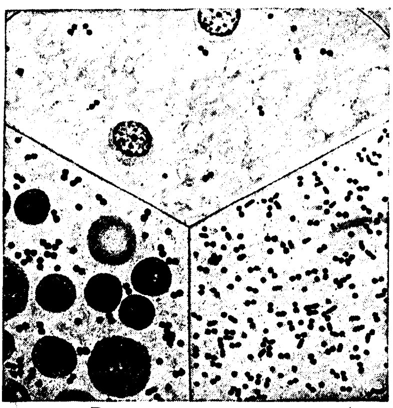


FIG. 211.—*Brucella melitensis.* *A*, smear of milk from an infected goat; *B*, spleen smear from a case of Malta fever; *C*, smear prepared from a young culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

1. The cause of contagious abortion in cattle. The same effects are produced in mares, sheep, rabbits, and guinea pigs. Causes undulant fever (brucellosis) in man.

2. Tissues, blood, milk, and urine of infected animals.

3. Drinking milk of infected animals; contact with infected animals.

4. Uncertain.

5. Disease controlled by the destruction or isolation of infected animals; pasteurization of all milk. The disease is seldom fatal.

Brucella melitensis.—Short, oval rods 0.3 to 0.4μ in length. Occur singly and in pairs, occasionally in chains. Nonmotile, nonacid-fast, and microaerophilic. Gram-negative.

1. The cause of undulant fever (brucellosis) in man and abortion in goats. May infect cows and be excreted in their milk. Infectious for all domestic animals (Fig. 211).

2. Tissues, blood, milk, and urine of infected animals.

3. Drinking milk of infected animals; contact with infected animals.

4. Uncertain.

5. Disease controlled by the destruction or isolation of infected animals; pasteurization of all milk. The disease is seldom fatal.

Brucella suis.—Short, oval rods 0.3 to 0.4μ in length. Occur singly and in pairs, occasionally in chains. Nonmotile and nonacid-fast. Gram-negative.

1. The cause of abortion in swine and frequently of undulant fever (brucellosis) in man. Also infectious for horses, dogs, cows, monkeys, and laboratory animals.

2. Tissues, blood, milk, and urine of infected animals.

3. Contact with infected animals; drinking milk of infected animals.

4. Uncertain.

5. Disease controlled by the destruction or isolation of infected animals; pasteurization of all milk. The disease is seldom fatal.

CLOSTRIDIUM

Clostridium botulinum.—Rods 0.5 to 0.8 by 3.0 to 8.0μ, with rounded ends; occur singly, in pairs, and in short to occasional long chains. Motile. Spores oval, central, subterminal to terminal. Gram-positive.

1. The cause of botulism. Pathogenic for animals. Produces a powerful exotoxin which is neurotoxic both on injection and feeding.

2. Contaminated smoked, pickled, or canned foods.

3. Consumption of smoked, pickled, or canned foods containing the exotoxin.

4. Passive immunity with antitoxin.

5. Symptoms are due to the ingestion of foods containing a powerful exotoxin elaborated by the specific organism. The exotoxin is neutralized by homologous antitoxin and by *C. parabotulinum* Type *B* antitoxin.

Clostridium botulinum Type C.—This may be regarded as a variety of *C. botulinum*, the two organisms being similar in their morphological and cultural characters. Rods 0.5 to 0.8 by 3.0 to 6.0μ, commonly slightly curved.

1. Produces botulism in man and limberneck in chickens.

2. Contaminated smoked, pickled, or canned foods.

3. Consumption of smoked, pickled, or canned foods containing the organism.

4. Passive immunity with antitoxin

5. Symptoms are due to the ingestion of foods containing a powerful exotoxin elaborated by the organism. The exotoxin is neutralized by homologous Type C antitoxin.

Clostridium parabotulinum.—Rods 0.5 to 0.8 by 3.0 to 8.0μ with rounded ends; occur singly, in pairs, and in short chains. Motile. Spores oval, subterminal, and cause a bulging of the rods. Gram-positive.

1. Produces botulism in man and animals.

2. Chiefly from smoked, pickled, nonacid canned foods, and from silage.

3. Consumption of smoked, pickled, or canned foods, and silage containing the exotoxin.

4. Passive immunity with antitoxin.

5. Symptoms are due to the ingestion of foods containing a powerful exotoxin elaborated by the organism. The exotoxin is neutralized by the homologous antitoxin.

Clostridium chauvoei.—Long rods 1.0 by 3.0 to 8.0μ, occurring singly, in pairs, and in short chains. Usually stain more heavily at the poles. Motile. Anaerobic. Spores oval, excentric to subterminal, causing a bulging of the rods. Gram-positive.

1. The cause of blackleg, black quarter, or symptomatic anthrax in cattle and other animals.

2. Organism found in the soil.

3. Disease due to entrance of the organism into the broken skin. It may follow shearing, docking, parturition, etc., but usually appears spontaneously.
4. Active immunity follows vaccination of animals.
5. Immune serum has given favorable results.

Clostridium histolyticum.—Rods 0.5 to 0.7 by 3.0 to 5.0μ. Occur singly and in pairs. Motile. Anaerobic. Spores oval, subterminal, and cause a bulging of the rods. Gram-positive.

1. Isolated from war wounds. Causes a necrosis of tissue. Pathogenic for small laboratory animals. Produces a cytolytic exotoxin which causes local necrosis and sloughing on injection. Not toxic on feeding.
2. Organism found in the soil.
3. Disease due to entrance of the organism into the broken skin where it multiplies and produces a soluble exotoxin.
4. Passive immunity with antitoxin.
5. The exotoxin can be neutralized by the injection of the homologous antitoxin.

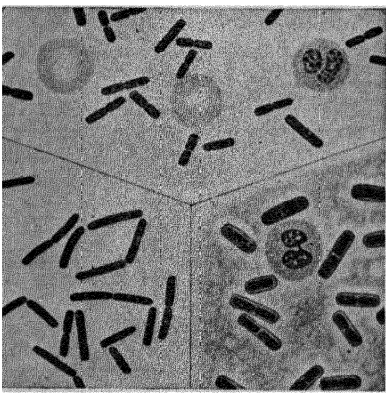


FIG. 212.—*Clostridium perfringens.* *A*, smear of the exudate from a case of human gas gangrene; *B*, smear from a young anaerobic agar culture; *C*, another smear from a case of gas gangrene. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

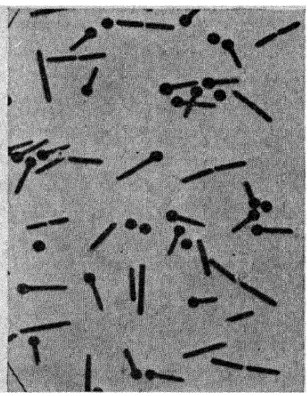


FIG. 213.—*Clostridium tetani.* Smear from a 72-hr. glucose agar stab culture. The spores are round and situated at one end of the rod. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Clostridium perfringens (welchii).—Rods short, thick, 1.0 to 1.5 by 4.0 to 8.0μ, occur singly and in pairs, less frequently in short chains. Nonmotile. Spores oval, central to excentric, do not cause a bulging of the rods. Capsules produced. Anaerobic. Gram-positive.

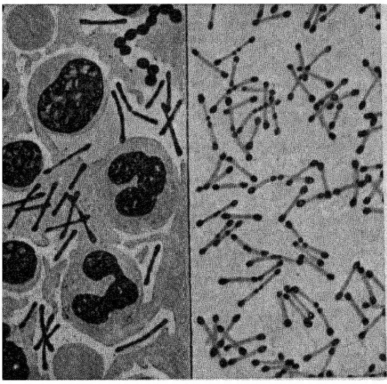
1. The cause of gas gangrene. Pathogenic for guinea pigs, pigeons, and mice (Fig. 212).
2. Organism found in soil, feces, sewage, milk, and in gas gangrene.
3. Disease due to entrance of organism into the broken skin where it multiplies and produces a soluble exotoxin.
4. Passive immunity with antitoxin.
5. The exotoxin can be neutralized by the injection of the homologous antitoxin.

Clostridium tetani.—Rods 0.4 to 0.6 by 4.0 to 8.0μ, with rounded ends, occur singly, in pairs, and in chains. Motile. Spores round, terminal, and cause a bulging of the rod. Anaerobic. Gram-positive.

1. The causative agent of tetanus (Fig. 213).
2. Organism found in soil, and in human and horse intestines and feces.
3. Disease due to entrance of organism into the broken skin where it multiplies and produces a potent exotoxin. Toxin intensely toxic on injection but not on feeding.
4. Passive immunity with antitoxin.
5. The exotoxin can be neutralized by the injection of the homologous antitoxin.

CORYNEBACTERIUM

Corynebacterium diphtheriae Types I, II, III, IV and V.—Rods vary greatly in size, 0.3 to 0.8 by 1.0 to 8.0μ, and occur singly. Rods are straight or slightly curved, frequently club-shaped at one or both ends. Stained rods show the presence of metachromatic granules. Nonmotile. Gram-positive.



A B

FIG. 214.—*Corynebacterium diphtheriae.* *A*, smear prepared from a throat swab taken from a case of diphtheria; *B*, smear from a 12-hr. culture on Loeffler's blood serum medium. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

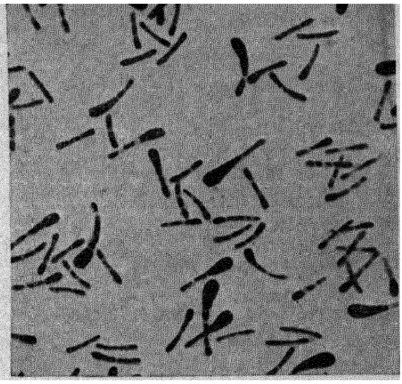


FIG. 215.—*Corynebacterium diphtheriae.* Smear prepared from a 5-day old agar slant culture showing involution or club-shaped forms frequently encountered in old cultures. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

1. The cause of diphtheria in man; may be transmitted to guinea pigs, kittens, and rabbits (Figs. 214 and 215).
2. Commonly from the membrane in the pharynx, larynx, trachea, and nose in human diphtheria; from the healthy pharynx and nose of carriers. Occasionally found infecting the nasal passages and wounds in horses.
3. Disease spread from person to person by fingers, or articles, such as eating utensils, toys, pencils, handkerchiefs containing nasal discharges and saliva, and by inhalation of droplets expelled from the throat during coughing or sneezing. The organism produces a powerful exotoxin for which an antitoxin may be prepared.
4. Good acquired immunity; artificial active immunity with toxoid (modified diphtheria toxin); passive with antitoxin.
5. Isolation of suspected cases of diphtheria. Active immunization of susceptible individuals with toxoid. Administration of diphtheria antitoxin to clinical cases.

DIPLOCOCCUS

Diplococcus pneumoniae.—Organisms 0.5 to 1.25μ, oval or spherical in shape. Occur in pairs, occasionally in short chains. The distal ends of each pair are pointed or lancet-shaped. Produce capsules. Gram-positive.

1. The commonest cause of lobar pneumonia. Thirty-two types are recognized on the basis of agglutination with immune serums (Figs. 216 and 217).

2. Sputum, blood, and exudates in pneumonia; cerebrospinal fluid in meningitis; mastoiditis; saliva from respiratory tract of normal individuals.

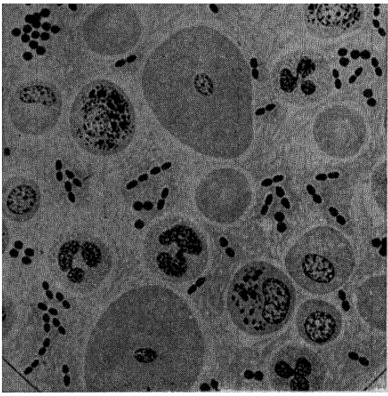


FIG. 216.—*Diplococcus pneumoniae.* Smear of sputum from a case of lobar pneumonia. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

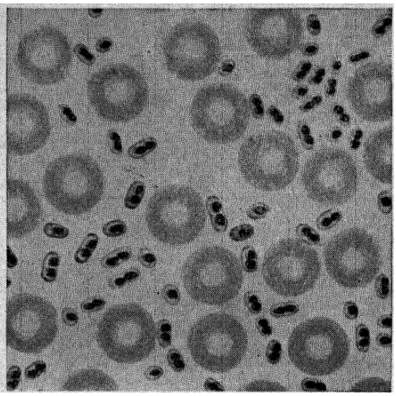


FIG. 217.—*Diplococcus pneumoniae.* Smear prepared from the heart blood of an infected mouse. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

3. Direct contact with infected person or carrier; inhalation of droplets expelled from the throat during coughing or sneezing.

4. Immunity relatively slight and of short duration. Prophylactic immunization by the use of a vaccine prepared by chemical treatment (capsular substance) produces good results.

5. Isolation of suspected cases of pneumonia. Injections of the proper therapeutic antiserum; employment of drugs of the sulphonamid type (sulfanilamid, sulfapyridine, sulfaphthiazole, etc.).

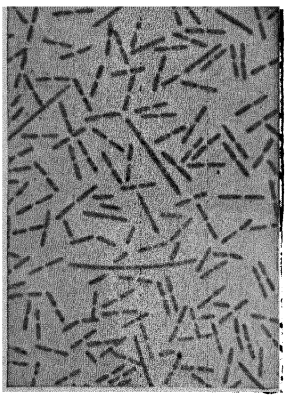


FIG. 218.—*Eberthella typhosa.* Smear from a 24-hr. agar slant culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

EBERTHELLA

Eberthella typhosa.—Eleven types recognized. Rods 0.6 to 0.7 by 2.0 to 3.0μ, occurring singly, in pairs, and occasionally in short chains. Facultative anaerobic. Motile. Gram-negative.

1. The causative agent of typhoid fever in man. Pathogenic for laboratory animals on subcutaneous or intravenous inoculation (Fig. 218).

2. Feces and urine of typhoid patients or carriers.

3. Disease transmitted through personal contact with patients or carriers. Foods contaminated by fingers of typhoid patients or carriers. The most common source of typhoid outbreaks is through milk, contaminated by a dairy worker. Oysters and other shellfish, grown in sewage-polluted waters, may harbor the organism. Water-borne epidemics, due to sewage contamination, sometimes occur.

4. Permanent acquired immunity after recovery from an infection. Active artificial immunity after vaccination.

5. Disease prevented and controlled by the following measures: Efficient sewage disposal system; pure water supply; pure milk supply; sanitary control of foods, especially milk products and shellfish; periodic examination of individuals who handle foods for public consumption; and extermination of flies.

ERYSIPELOTHRIX

Erysipelothrix rhusiopathiae.—Slender rods 0.2 to 0.3 by 0.5 to 1.5μ, occurring singly and in chains. Microaerophilic. Nonmotile. Gram-positive.

1. The cause of swine erysipelas. Transmissible to gray and white mice, pigeons, and rabbits. Infection has been transferred to man by accidental inoculation.
2. Organism found in slimy surfaces of fish where it lives as a harmless parasite. Soil, water, and manure are frequently contaminated. Organism always present in feces and urine of infected animals. Persons who handle diseased animals may be carriers.
3. Natural infection believed to take place by ingestion or through the broken skin.
4. Permanent acquired immunity.
5. All infected animals should be segregated and given immune serum. Contaminated buildings should be thoroughly disinfected. Polluted pastures and troughs should not be used for a long period of time. Healthy animals may be immunized by the injection of an immune serum followed two days later by the inoculation of a known amount of a virulent culture of the organisms. Sometimes the animals are given a second injection of the vaccine after an interval of 14 days.

FUSOBACTERIUM

Fusobacterium plauti-vincenti.—Rods 0.5 to 1.0 by 8 to 16μ, occurring in pairs, short curved chains, or long spirillum-like threads. Organisms that occur in pairs have blunt ends together and outer ends pointed. Rods show from 2 to 6 deeply staining granules. Anaerobic. Nonmotile. Gram-negative.

1. Organism associated with *Borrelia vincentii* in Vincent's angina, an acute infection of the tonsils, or neighboring parts, and characterized by the appearance of a pseudomembranous inflammation followed by ulceration (Fig. 210).
2. Found in deposit on teeth; the oral cavity.
3. The disease is not ordinarily communicable. Under unusual conditions of crowding, such as may prevail among soldiers, the infection may become transmissible. The disease appears to be associated with a state of lowered resistance. The tonsillar ulceration occurs often in individuals whose resistance has been lowered by diseases such as measles, tuberculosis, diabetes, and scarlet fever.
4. No immunity.
5. The disease usually yields to local treatment. The most effective drug for this purpose appears to be salvarsan, an arsenic compound employed in the treatment of syphilis.

GAFFKYA

Gaffkya tetragena.—Spheres 0.6 to 0.8μ in diameter. Organisms grow in clusters of fours (tetrads) surrounded by a capsule when in body fluids. Nonmotile. Gram-positive.

1. Found on mucous membrane of the respiratory tract. Isolated from sputum in tuberculosis; also present in air and on the skin. Pathogenic for mice, guinea pigs, and rabbits (Fig. 219).
2. Present in sputum, air, and on skin.

3. Transmitted through discharges from throat; by inhalation of droplets expelled during coughing and sneezing.
4. No immunity.
5. Isolation of suspected cases.

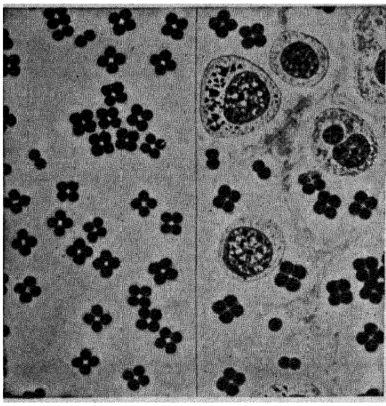
A  B

FIG. 219.—*Gaffkya tetragena*. *A*, smear from a 24-hr. agar slant culture; *B*, smear of sputum from a case of chronic tuberculosis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

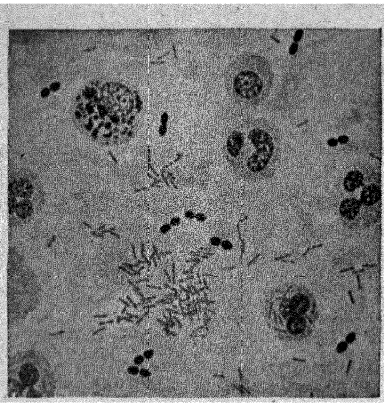


FIG. 220.—*Hemophilus influenzae*. Smear of sputum from a case of influenzal pneumonia. The oval-shaped cells are *Diplococcus pneumoniae*. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

HEMOPHILUS

Hemophilus pertussis.—Short, oval rods 0.2 to 0.3 by 0.5 to 2.0μ, occurring singly, sometimes in pairs. Show a tendency to bipolar staining. Nonmotile. Gram-negative.

1. Believed to be the cause of whooping cough in man.
2. Isolated from the respiratory tract in active cases of whooping cough, especially by the cough-plate method.
3. Contact with infected persons or infected articles. Disease easily spread among children by personal contact.
4. Permanent acquired immunity after recovery from the infection. Artificial active immunity after vaccination.
5. Isolation of infected individuals. Vaccination of those exposed to the disease. Vaccine has also been employed in the treatment of the infection.

Hemophilus influenzae.—Very small rods 0.2 to 0.3 by 0.5 to 2.0μ, occurring singly, in pairs, occasionally in short chains, and sometimes in the form of long threads. Show a marked tendency to bipolar staining. Some strains produce capsules. Nonmotile. Gram-positive.

1. Isolated from cases of influenza; commonly present in blood and spinal fluid in meningitis of infants; the cause of acute infectious conjunctivitis or "pink eye" (Figs. 220 and 221).
2. Discharges from the nose and throat of, or contact with, an infected individual.
3. Contact with an infected person; the use of towels and other infected articles; the inhalation of droplets expelled during coughing and sneezing.
4. Doubtful.
5. Isolation of infected individuals; avoidance of overcrowding.

Hemophilus duplex.—Short rods 0.4 to 0.5 by 2.0μ, occurring singly, in pairs, and in short chains. Ends rounded or square in the chains. Nonmotile. Gram-negative.

1. The cause of subacute infectious **conjunctivitis or angular conjunctivitis.** Isolated from conjunctiva (Fig. 222).

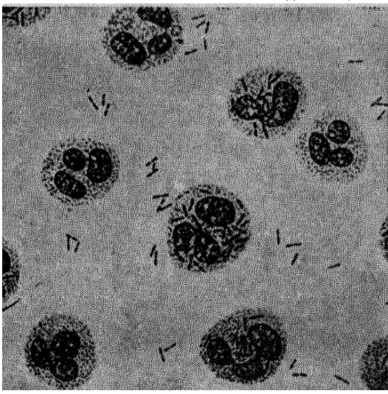


Fig. 221.—*Hemophilus influenzae.* Smear of pus from a case of acute conjunctivitis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

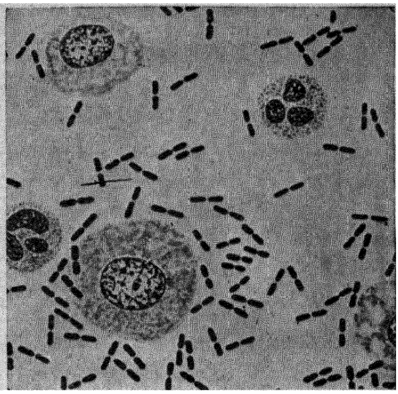
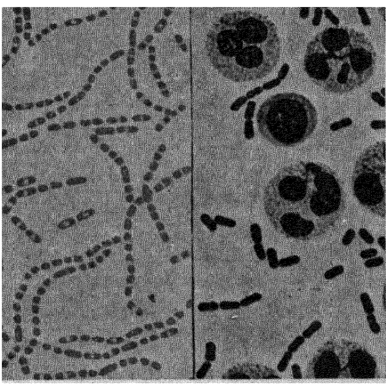
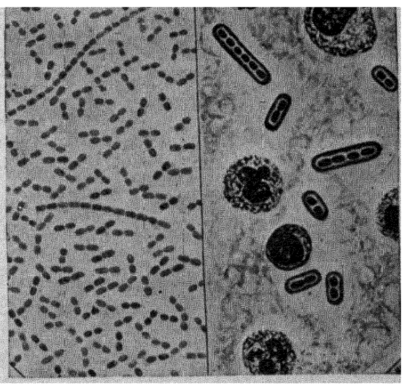


Fig. 222.—*Hemophilus duplex.* Smear of lachrymal secretion from a case of chronic conjunctivitis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)



A *B*

Fig. 223.—*Hemophilus ducreyi,* the causative organism of soft chancre or chancroid. *A,* smear from a young blood agar culture; *B,* smear from a soft chancre. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)



A *B*

Fig. 224.—*Klebsiella pneumoniae* or Friedländer's bacillus. *A,* smear from a culture; *B,* smear of sputum. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

2. Discharges from conjunctivae.
3. Contact with discharges or with infected person.
4. No acquired immunity.
5. Isolation of patient; disinfection of the affected area.

Hemophilus ducreyi.—Small rods 0.5 by 1.5 to 2.0μ, with rounded ends, occurring singly and in short chains. Nonmotile. Gram-negative.

1. The cause of soft chancre or chancroid (Fig. 223).
2. Discharges from ulcerated areas.
3. Infection usually direct from individual to individual, particularly during coitus.
4. Probably no immunity.
5. Chancroid is spread almost entirely by sexual contact. It is not a serious disease and usually yields readily to local treatment.

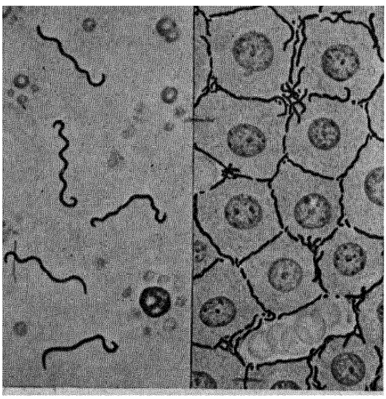
KLEBSIELLA

Klebsiella pneumoniae.—Rods 0.3 to 0.5 by 5.0μ, ends rounded, often 4 to 5 times as long as broad, occurring singly and in pairs. Produce capsules. Nonmotile. Gram-negative.

1. Isolated from the lungs in lobar pneumonia. Associated with pneumonia and other inflammations of the respiratory tract (Fig. 224).
2. Buccal and nasal discharges of infected persons or carriers; articles contaminated with such discharges.
3. Direct contact with infected person or carrier.
4. Immunity relatively slight and of short duration.
5. Isolation of infected persons; concurrent disinfection.

LEPTOSPIRA

Leptospira icterohaemorrhagiae.—Cells cylindrical 0.25 to 0.3 by 6 to 9μ, ends pointed. Amplitude of spirals 0.4 to 0.5μ, regular, rigid. Depth of spiral 0.3μ, regular. One or more wavy curves throughout the length of the organism. One or both ends bent into a hook. No flagella. Highly motile end portion well developed in the last 6 or 8 spirals. Transverse division. Aerobic.



A B

Fig. 225.—*Leptospira icterohaemorrhagiae*, the organism responsible for Weil's disease or infectious jaundice in man. *A*, smear of urine; *B*, section of liver from an infected rat. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

1. The causative agent of infectious jaundice (Weil's disease). Found in the blood of dogs and wild rats (Fig. 225).
2. Infected rats and dogs are the natural reservoir. Water and soil become contaminated by urine of these animals.
3. Infection in man probably occurs through rubbing contaminated soil into the skin, eyes, and nose or from swallowing contaminated water.
4. Long period of immunity after recovery from an attack.
5. Prevention of disease consists in the drainage of infected areas and the suppression or exclusion of rats. Vaccine has been employed with promising results. The most promising treatment appears to be the use of an immune horse serum.

Leptospira icteroides.—Organism similar to *L. icterohaemorrhagiae* in its morphological characteristics. Cells 0.2 to 0.25 by 4 to 9μ, tapering toward extremities, which are sharply pointed. Multiplication by transverse division.

1. Believed by Noguchi to be the cause of yellow fever. Organism not present in patients suffering from yellow fever in Africa.
2. Blood from infected individuals.

3. Transmissible to guinea pigs. Disseminated by the bite of the yellow fever mosquito *Aëdes egypti* (*Stegomyia calopus*).

MALLEOMYCES

Malleomyces mallei.—Rods 0.5 to 1.0 by 2.0 to 5.0μ, with rounded ends, occurring singly, in pairs, in groups, and sometimes in filaments. Branching involution forms appear on glycerin agar. Bipolar staining. Nonmotile. Gram-negative.

1. The cause of glanders in horses, sheep, goats, and man. Disease transmissible to dogs, cats, rabbits, and guinea pigs (Fig. 226).

2. Isolated from the liver and spleen of a horse. Produces lesions in the liver, spleen, pancreas, lungs, kidneys, ovaries, testes, nasal membranes, and other mucous surfaces. Large nodules of pus always present with the lesions.

3. Infection usually occurs from mucous membranes and breaks in the skin. It passes from horse to horse mostly by infected nasal discharges, more rarely from ulcerous discharges.

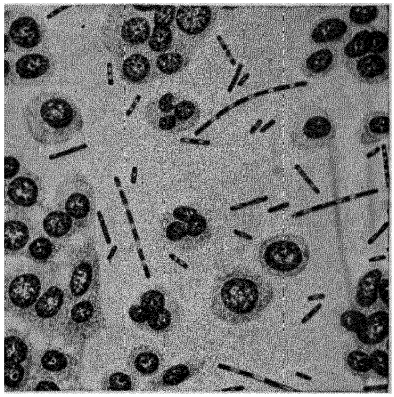


FIG. 226.—*Malleomyces mallei.* Smear from a lymphatic gland of an infected guinea pig. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

4. Poor active immunity. Horses easily contract the disease a second time.

5. Diseased animals should be segregated and slaughtered to prevent spread of disease to healthy animals. The stables should be thoroughly disinfected. No satisfactory method of treatment.

MYCOBACTERIUM

Mycobacterium tuberculosis var. hominis.—Rods 0.3 to 0.6 by 0.5 to 4.0μ, straight or curved, occurring singly and sometimes in threads. Under some conditions cells are swollen, clavate, or even branched. Rods may stain solidly or show a banded or beaded appearance. Acid-fast. Gram-positive.

1. The cause of tuberculosis in man, monkey, dog, and parrot. Experimentally very pathogenic for guinea pigs, but not for rabbits, cats, goats, oxen, or domestic fowls (Fig. 227).

2. Discharges from lesions; chiefly from sputum.

3. Transmitted by inhaling droplets expelled during coughing, sneezing, talking, or singing; by contact, dust, flies, feces, etc.

4. Generally none. Resistance to infection increased by improved living conditions.

5. Avoid overcrowding; improve living and working conditions. Isolation of infected individuals. Cure based chiefly on rest, good food, fresh air, sunshine, and freedom from worry.

Mycobacterium tuberculosis var. bovis.—Rods shorter and more plump than the human type, ranging from 1.0 to 1.5μ. Short rods often mixed with larger forms. Cells may stain solidly or show a banded or beaded appearance. Acid-fast. Gram-positive.

1. The cause of tuberculosis in cattle. May be transmitted to man and domestic animals. Pathogenic for ox, man, monkey, goat, sheep, pig, cat, parrot, cockatoo, and other birds. Experimentally highly pathogenic for rabbit and guinea pig. More highly pathogenic for animals than the human type (Fig. 228).

2. Tuberculous cows may eliminate the organisms in the feces, urine, milk, etc.

3. Infection spread to healthy cows through milk, urine, and feces of diseased cows. Children, particularly those under 5 years of age, may become infected by drinking contaminated milk from diseased cows.

4. Generally none.

5. Destruction of diseased animals to prevent spread to healthy animals. Vaccination of healthy animals with a special vaccine known as B. C. G. Pasteurization of all milk.

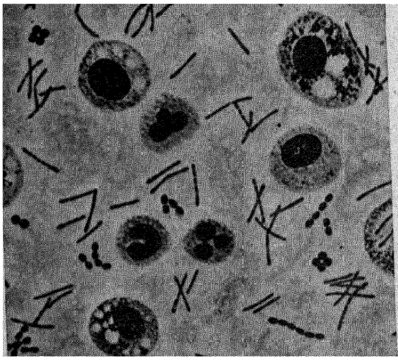


FIG. 227.—*Mycobacterium tuberculosis* var. *hominis*. Smear of sputum from a case of pulmonary tuberculosis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

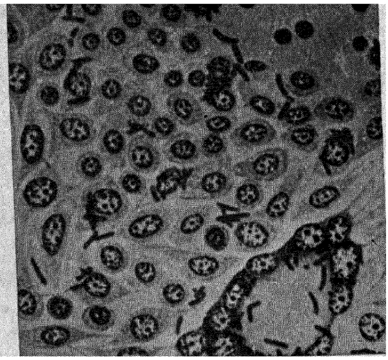


FIG. 228.—*Mycobacterium tuberculosis* var. *bovis*. Section of udder of cow suffering from chronic tuberculosis. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Mycobacterium paratuberculosis.—Plump rods 1.0 to 2.0μ in length. The plump forms stain uniformly but occasionally the longer rods show banded segments. Nonmotile. Acid-fast. Gram-positive.

1. The cause of Johne's disease, a chronic diarrhea in cattle, and sheep. Disease not transmissible to guinea pigs, rabbits, rats, and mice.

2. Organisms found in feces of infected animals, in contaminated water, food, pastures, and bedding.

3. Natural infection occurs from consuming infected food, water, grass containing fecal material, etc.

4. Generally none.

5. Isolation or destruction of diseased animals. Thorough disinfection of all buildings. Dung and fodder should be burned. Contaminated pastures should be plowed under and allowed to rest for at least one year. No specific treatment.

Mycobacterium leprae.—Rods 0.3 to 0.5 by 1.0 to 8.0μ, with parallel sides and rounded ends. Cells may stain solidly or show a banded or beaded appearance. Sometimes curved forms may be present. Organisms usually arranged in parallel bundles. Club-shaped forms, single, double, coccoid, and chains have been described. Nonmotile. Acid-fast. Gram-positive.

NEISSERIA

Neisseria gonorrhoeae.—Spherical cells 0.6 to 1.0μ in diameter, occurring singly and in pairs. The sides are flattened where they come in contact. Nonmotile. Gram-negative.

1. The cause of gonorrhoeal infection in man (Fig. 229).
2. Purulent venereal discharges, blood, pus from infections of the conjunctiva and joints.
3. Transmissible by direct contact; sexual intercourse.
4. None.
5. No specific method of treatment known. Treatment usually consists in the use of germicides, vaccines, and diathermy. Public health clinics have been established in most cities to treat infected individuals.

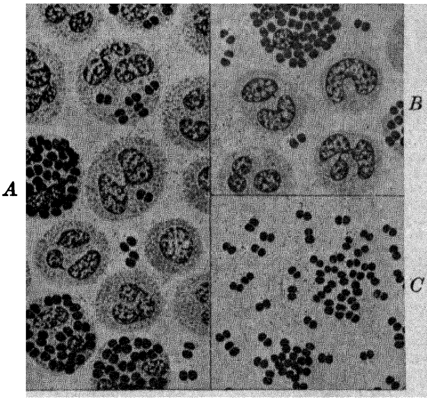


FIG. 229.—*Neisseria gonorrhoeae*, the causative organism of gonorrhea. *A*, a smear of pus showing the presence of the organism within the polymorphonuclear leucocytes; *B*, same as *A* but stained differently; *C*, smear from a pure culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

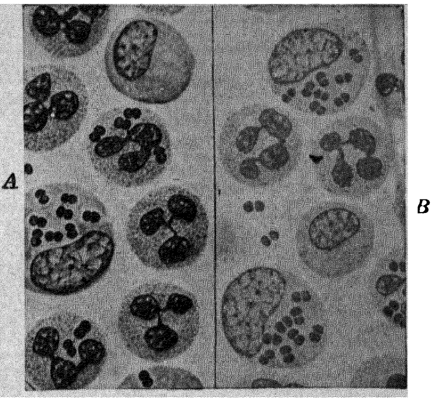


FIG. 230.—*Neisseria intracellularis*, the cause of cerebrospinal fever or meningococcal meningitis. *A*, smear of sediment from a centrifugalized specimen of cerebrospinal fluid. *B*, same as *A* but stained differently. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Neisseria intracellularis Types I, II, III, and IV.—Spherical cells 0.6 to 0.8μ in diameter, occurring singly, in pairs, and occasionally in tetrads. The sides are flattened where they come in contact. Nonmotile. Gram-negative.

1. The causative agent of epidemic meningitis.
2. Organisms found in nasopharynx, blood, cerebrospinal fluid, conjunctiva, pus from joints, etc., of persons suffering from the disease (Fig. 230).
3. The organisms are passed from person to person by droplet infection. Organisms very easily killed when outside of the body and probably never reach a new individual except by direct personal contact. Disease easily spread by overcrowding such as prevails in army camps.
4. Immunity acquired after an infection. Duration uncertain. Passive immunity by means of antiserum.
5. Isolation of infected persons; prevention of overcrowding; infected individuals treated by injections of antimeningococcus serum into the cerebrospinal canal after first removing the purulent spinal fluid.

PASTEURELLA

Pasteurella pestis.—Rods 1.0 by 2.0μ, occurring singly. Polar staining. Nonmotile. Gram-negative.

1. The cause of plague in man, rats, ground squirrels, and other rodents. Transmissible to mice, guinea pigs, and rabbits.

2. Organism found in buboes, blood, pleural effusion, spleen, and liver of infected persons and rodents. In addition organism present in the sputum in cases of pneumonic plague (Fig. 231).

3. Transmitted from rat to rat and from rat to man by infected rat fleas, the most important of which is *Xenopsylla cheopis.* The flea becomes infected by feeding on a diseased rat. The flea next feeds on a person and at the same time deposits feces and possibly some regurgitated blood. The bacilli are then rubbed into the broken skin by scratching.

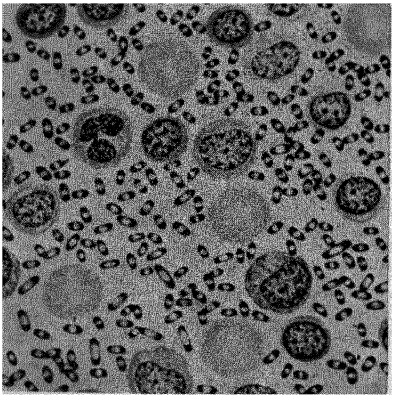


FIG. 231. *Pasteurella pestis,* the cause of bubonic, pneumonic, and septicaemic plague in man. Smear prepared from a bubo. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

4. Lasting acquired immunity following recovery from infection. Acquired immunity follows the administration of plague vaccine.

5. Rat extermination, rat proofing, and other necessary measures. Isolation of infected individuals. Use of vaccine and immune serum has proved of value.

Pasteurella tularensis.—Rods 0.2 by 0.3 to 0.7μ, occurring singly. Show bipolar staining. Pleomorphic. Nonmotile. Gram-negative.

1. The cause of tularemia in man. Infectious for rabbits, guinea pigs, rats, gray mice, and ground squirrels.

2. Organisms isolated from ground squirrels. Found in lesions in man and animals with natural or experimental infection, especially in spleen and liver.

3. Transmitted from wild animals to man by contact with infected animals or by the bite of horse flies, ticks such as *Dermacentor andersoni* and *D. occidentalis,* and possibly other biting insects. Hunters, cooks, and butchers may contract the disease during the rabbit-hunting season.

4. Permanent immunity after recovery from the disease.

5. Care in handling and dressing wild rabbits; thorough cooking of flesh.

SALMONELLA

Salmonella schottmuelleri.—Rods 0.6 to 0.7 by 2.0 to 3.0μ, occurring singly and in pairs. Motile. Gram-negative.

1. The cause of paratyphoid (enteric) fever in man. Not naturally found in animals.

2. Feces and urine of infected persons and carriers.

3. Transmitted by direct contact, by food and water, by insects, and by carriers.

4. Good acquired immunity. Artificial active immunity by means of vaccines.

5. Food and water sanitation; care of carriers; isolation of infected individuals and disinfection of discharges, clothing, etc.

Salmonella paratyphi.—Rods 0.6 by 3.0 to 4.0μ, occurring singly. Motile. Gram-negative.

1. The cause of paratyphoid (enteric) fever in man. Probably not a natural pathogen of animals.
2. Feces and urine of infected persons and carriers.
3. Transmitted by direct contact, by food and water, by insects, and by carriers.
4. Good acquired immunity. Artificial active immunity by means of vaccines.
5. Food and water sanitation; care of carriers; isolation of infected individuals and disinfection of discharges, clothing, etc.

Salmonella typhimurium.—Rods 0.5 by 1.0 to 1.5μ, occurring singly. Motile. Gram-negative.

1. A natural pathogen for mice, rats, guinea pigs, sheep, pigs, parrots, chickens, ducks, turkeys, pigeons, and canaries. Produces food poisoning in man.
2. Feces and urine of infected persons and carriers.
3. Transmitted by direct contact, by food and water, by insects, and by carriers.
4. Good acquired immunity.
5. Food and water sanitation; care of carriers; disinfection of discharges, clothing, etc.

Salmonella enteritidis.—Rods 0.6 to 0.7 by 2.0 to 3.0μ, occurring singly, in pairs, and sometimes in short chains. Motile. Gram-negative.

1. The cause of food poisoning in man. Organism produces natural infections in domestic and wild animals.
2. Feces and urine of infected persons and carriers.
3. Transmitted by direct contact, by food and water, by insects, and by carriers.
4. Good acquired immunity.
5. Food and water sanitation; care of carriers; disinfection of discharges, clothing, etc.

SHIGELLA

Shigella dysenteriae.—Rods 0.4 to 0.6 by 1.0 to 3.0μ, occurring singly. Nonmotile. Gram-negative.

1. A cause of dysentery in man and monkeys (Fig. 232).
2. Bowel discharges of infected individuals; food and water.
3. Transmitted by personal contact, by food and water, by flies and other insects, and by carriers.
4. Slight acquired immunity.
5. Food and water sanitation; avoidance of overcrowding; disinfection of discharges and clothing of patients; vaccination has been practiced with some success. Treatment of carriers.

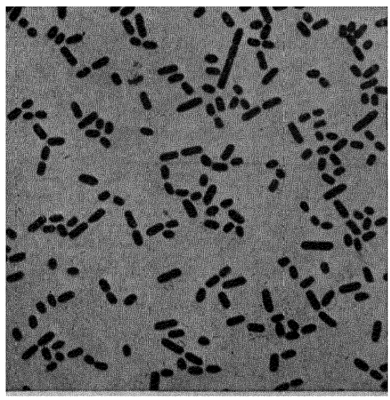


FIG. 232.—*Shigella dysenteriae.* Smear from a 24-hr. culture. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Shigella paradysenteriae.—Rods 0.4 to 0.6 by 1.0 to 3.0μ, occurring singly. Nonmotile. Gram-negative.

1. A cause of dysentery in man and summer diarrhea in children.
2. Bowel discharges and clothing of infected individuals; food and water.
3. Transmitted by personal contact, by food and water, by flies and other insects, and by carriers.

4. Slight acquired immunity.
5. Food and water sanitation; avoidance of overcrowding; disinfection of discharges and clothing of patients.

Shigella sonnei Types I and II.—Rods. Nonmotile. Gram-negative.
1. A cause of mild dysentery in man and summer diarrhea in children.
2. Bowel discharges of infected individuals; food and water.
3. Transmitted by personal contact, by food and water, by flies and other insects, and by carriers.
4. Very slight acquired immunity.
5. Food and water sanitation; avoidance of overcrowding; disinfection of discharges and clothing of patients; treatment of carriers.

STAPHYLOCOCCUS

Staphylococcus aureus.—Spheres 0.8 to 1.0μ in diameter, occurring singly, in pairs, in short chains, and in irregular clusters. Nonmotile. Gram-positive.

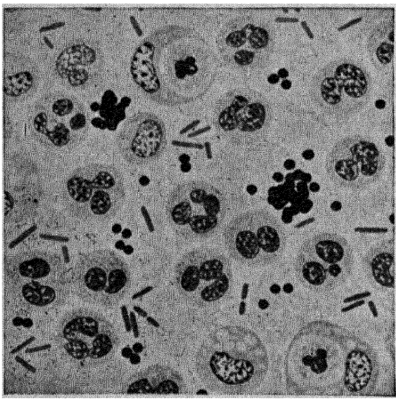


FIG. 233.—Staphylococci and *Pseudomonas aeruginosa*. Smear of pus showing the presence of organisms and white blood cells. The two organisms are frequently found together in pyogenic infections. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

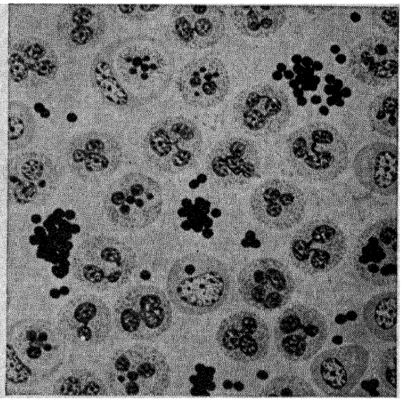


FIG. 234.—*Staphylococcus aureus*. Smear of pus showing organisms and white blood cells. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

1. Found on skin and mucous membranes. The causative organism of pimples, boils, abscesses, furuncles, suppuration in wounds, etc. (Figs. 233 and 234).
2. Pus, air, skin, contaminated clothing, food, water, etc.
3. Transmitted by entrance of organisms into a cut or break in the skin.
4. Very little, if any.
5. Aseptic surgery; destruction of soiled dressings by burning; oral and skin cleanliness.

Some pathogenic strains of *S. aureus* secrete several kinds of toxins. Culture filtrates are capable of (1) hemolyzing red blood cells (hemolysin), (2) destroying leucocytes (leucocidin), (3) causing death of animals when injected intravenously (tissue toxin), (4) causing food poisoning when ingested (enterotoxin), and (5) dissolving or digesting human fibrin (fibrinolysin).

Staphylococcus albus.—Spheres 0.6 to 0.8μ in diameter, occurring singly, in pairs, and in irregular clusters. Nonmotile. Gram-positive.

1. Found on skin and mucous membranes. The causative organism of pimples, boils, abscesses, furuncles, suppuration in wounds, etc.
2. Pus, air, skin, contaminated clothing, food and water, etc.
3. Transmitted by entrance of organisms into a cut or break in the skin.
4. Very little, if any.
5. Aseptic surgery; destruction of soiled dressings by burning; oral and skin cleanliness. Autogenous vaccines have been found helpful in some cases.

Staphylococcus aerogenes.—Spheres 0.6 to 0.8μ in diameter, occurring in pairs, in short chains, and in irregular clusters. Nonmotile. Gram-positive.

1. Found in natural cavities of the body, especially in the tonsils and in the female genital organs. The cause of puerperal fever.
2. Found in infected tonsils, discharges from body cavities, air, food, contaminated clothing, skin, etc.
3. Transmitted by contact; by entrance of organisms into a cut or break in the skin.
4. Very little, if any.
5. Aseptic surgery; destruction of soiled dressings by burning; oral and skin cleanliness.

STREPTOCOCCUS

Streptococcus pyogenes.—Cells spherical or oval 0.6 to 1.0μ in diameter, occurring in pairs or in chains. Capsule formation variable. Nonmotile. Gram-positive.

1. Found in human mouth, throat, respiratory tract, inflammatory exudates; produces septic sore throat, septicemia, erysipelas, scarlet fever, puerperal fever, and many other infections (Fig. 235).
2. Organisms spread by contaminated milk, pus, sputum, nasal discharges, etc.
3. Transmitted by direct contact; by inhaling droplets expelled during coughing, sneezing, or talking; consumption of contaminated milk; etc.
4. Very little, if any, and of temporary duration.
5. Pasteurization of milk; avoid contact with infected individuals or carriers; care in treating cuts and abrasions; disinfection of dressings, discharges, clothing, etc.

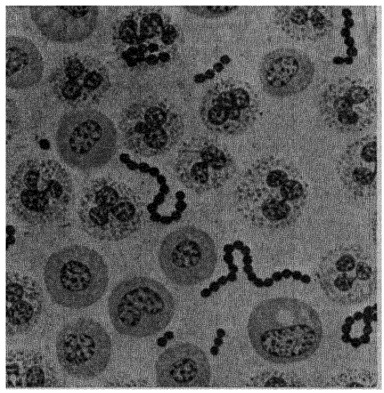


FIG. 235.—*Streptococcus pyogenes.* Smear from human pus showing the typical appearance of haemolytic streptococci together with polymorphonuclear and mononuclear leucocytes. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Culture filtrates of typical strains are capable of hemolyzing red blood cells. The soluble toxin is called a hemolysin. Two types of hemolysin are elaborated, one being oxygen-sensitive and the other oxygen-stable. On a blood agar plate the organisms produce a type of hemolysis generally referred to as beta hemolysis and possessing considerable diagnostic importance.

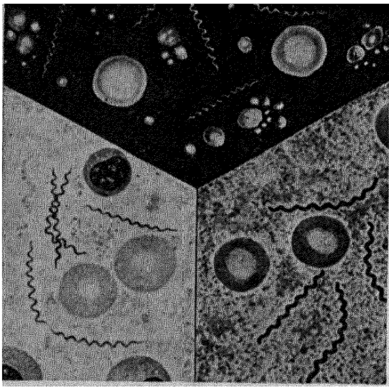
TREPONEMA

Treponema pallidum.—Cylindrical cells 0.25 to 0.3 by 6.0 to 14.0μ with pointed ends. Amplitude of spiral 1.0μ and regular. Depth of spiral 0.5 to 1.0μ and constant. Cell appears to consist of a spirally wound axial filament. Terminal spiral filament

present and easily seen in cultures. Multiplication transverse or possibly longitudinal also. Anaerobic.

1. The cause of syphilis in man (Fig. 236).
2. Discharges from lesions.
3. Direct personal contact with syphilitic individual; sexual intercourse. Transmitted by syphilitic mother to offspring.
4. No immunity.
5. Education in personal and sexual hygiene. Specific treatment consists of the injection of the arsenical known as arsphenamine or 606. Mercury and fever therapy also employed.

A



B C

FIG. 236.—*Treponema pallidum*, the causative organism of human syphilis. *A*, exudate from a primary sore, viewed by dark ground illumination; *B*, smear of material from a chancre; *C*, same as *B* but stained by a different technique. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

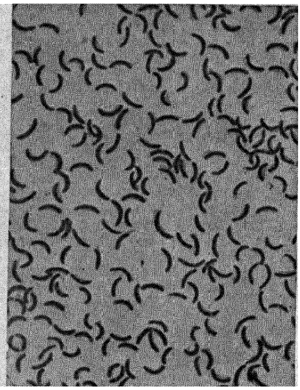


FIG. 237.—*Vibrio comma*, the causative agent of cholera in man. Smear from a 24-hr. agar slant culture. A chain of the curved rods presents a spiral formation. (*From Muir, "Bacteriological Atlas," E. and S. Livingstone, Edinburgh, Scotland.*)

Treponema pertenue.—Spirals 0.25 to 0.3 by 18 to 20μ. Anaerobic.

1. The causative organism of yaws or tropical frambesia. Transmissible to monkeys and to rabbits.
2. From ulcerated nodules on the skin.
3. Spread by contact. Transmission favored by nakedness and by unsanitary conditions. Flies can transmit the disease experimentally.
4. Recovery confers a lasting immunity.
5. Improved hygienic conditions an important factor in prophylaxis. Disease generally yields to injections of arsphenamine or 606.

VIBRIO

Vibrio comma.—Rods slightly curved measuring 0.4 to 0.6 by 1.5 to 3.0μ, occurring singly and in spiral chains. Motile. Gram-negative.

1. The cause of cholera in man (Fig. 237).
2. Bowel discharges, vomitus, carriers, food, and water.
3. Transmitted by food, water, contact with infected individuals, contaminated articles, flies, etc.
4. Recovery from the disease confers lasting immunity.

5. Disinfection of water; isolation of infected individuals; **disinfection of discharges**; use of clean water and food.

For further reading on the bacterial diseases of man and animals consult Bergey, Breed, Murray, and Hitchens (1939), Burdon (1939), Gay (1935). Topley and Wilson (1936), and Zinsser and Bayne-Jones (1939).

VIRUSES

The etiological agents of disease discussed in the preceding section can be seen with the aid of a microscope. The viruses, with few exceptions, are invisible under the highest power of the ordinary type of microscope. Their presence can be demonstrated only by the inoculation of the virus-containing material into a susceptible animal or plant. The viruses are found only in cellular material and their multiplication requires living cells for a culture medium. In neutral or slightly alkaline suspensions most viruses show a negative electrical charge. In this respect they behave similar to bacteria.

Nature of Viruses.—The exact nature of viruses is not clearly understood. Some believe that viruses are living organisms of infinitesimal size and develop by multiplication of preexisting forms. In support of this hypothesis is the work of Paschen (1906) who demonstrated the presence of elementary bodies in material from smallpox and vaccinia that he believed to be the etiological agent of the disease. Since that time elementary bodies have been shown to be present in a number of viruses.

Others believe that viruses are not living organisms but autocatalytic bodies capable of producing an injurious action in a susceptible plant or animal cell with the result that the cell is stimulated to produce more virus of the same kind. The cell is eventually destroyed after which the autocatalytic agent spreads to other cells of the host. Supporting this belief is the work of Stanley (1935) who isolated the virus of tobacco mosaic disease from infected tobacco and cucumber plants in the form of fine needle-shaped rods or crystals in a high state of purity, which were capable of reproducing the disease in healthy plants. Since that time other workers have isolated crystalline viruses from a number of diseased plants and have confirmed the findings of Stanley. The virus crystals have been shown to be composed of pure nucleoprotein molecules.

Perhaps both theories are correct Available evidence at present seems to point to the fact that the animal viruses may be living whereas some of the plant viruses, or possibly all of them, are nonliving, crystalline, protein molecules. All of the crystalline viruses have been obtained from plant cells. No worker has succeeded in crystallizing a virus from an animal cell.

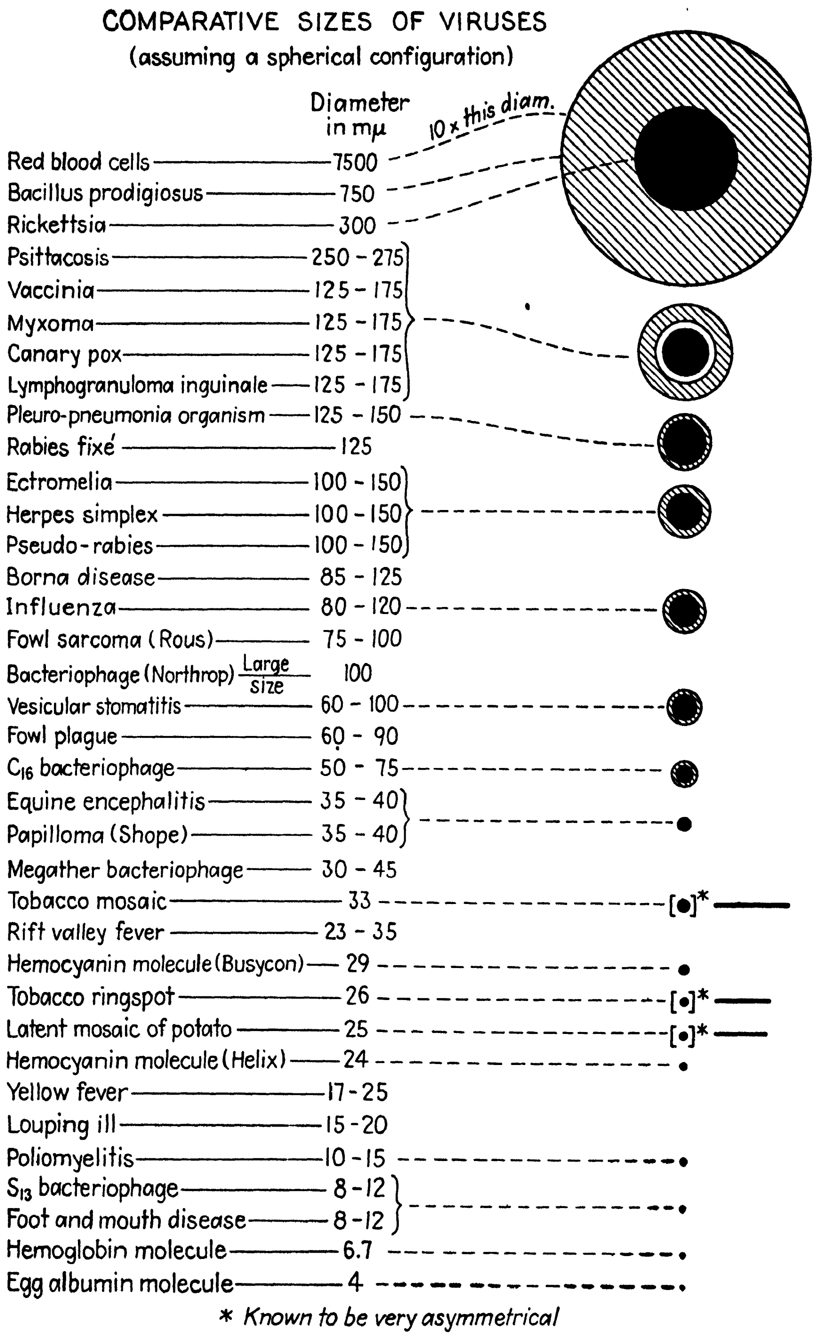


FIG. 238.—Relative sizes of a number of plant and animal viruses and protein molecules. (*After Stanley.*)

Size of Viruses.—Viruses show considerable variation in size. Some are as large as the smallest bacteria; others approach the dimensions of the smallest protein molecule. The relative sizes of a number of viruses, according to Stanley (1938), are given in Fig. 238. It is difficult to understand how a virus particle consisting of only one molecule can be considered to be a living organism capable of multiplication. It is equally as difficult to understand how a particle of psittacosis virus can be so large as to be visible microscopically and yet be considered to be a protein molecule or an autocatalytic, enzyme-like molecule manufactured by a living cell.

Cultivation of Viruses.—As has already been said, living cells of plant or animal are required for the successful propagation of plant or animal viruses. Although viruses multiply only in the presence of living cells, it does not follow that all viruses can be cultivated in this manner. Many viruses, especially those of animal origin, have not been successfully cultivated by this procedure. Some animal viruses may be cultivated in minced embryo medium, on the chorio-allantoic membrane of the developing chick, and in living tissue fragments embedded in plasma.

Plant viruses cannot be propagated in animal tissues. They require plant tissues for successful multiplication. Root-tip cultures are usually used for this purpose.

Paschen or Elementary Bodies.—The virus of vaccinia has been shown to be composed of small spherical bodies measuring about 0.15μ in diameter. They were first seen by Paschen (1906) and are generally referred to as Paschen bodies. They are sometimes called elementary bodies because the larger intracellular bodies seen in diseased cells probably consist of tightly packed masses of these small particles. These bodies are present in the lesions of the disease and also in the cells of tissues cultivated in vitro.

Parker and Rivers (1936) prepared large quantities of elementary bodies by grinding infected tissue with salt solution followed by mild centrifugation to sediment the coarser cell fragments. The supernatant fluid was removed and recentrifugated at high speed to throw down the elementary bodies. The particles were found to be highly infectious. Later Parker (1938) showed that the injection of a single elementary particle was sufficient to produce an infection. Elementary analyses of these particles showed that they were composed of fats, proteins, and carbohydrates.

Paschen bodies are capable of inciting the production of agglutinins when injected into animals. When a suspension of elementary bodies was filtered, the filtrate produced a visible precipitate when mixed with immune serum. This means that both agglutinins and precipitins are elaborated.

The two enzymes phosphatase and catalase have been shown to be present. Doubtless other enzymes are also present.

The foregoing results apparently add up to the conclusion that Paschen bodies may be considered to be living organisms, differing from spherical bacterial cells in being smaller and in lacking certain essential enzymes. The fact that multiplication of the virus particles occurs may be explained on the assumption that the Paschen bodies utilize the necessary enzymes present in the cells of the host. This may possibly explain the specificity of viruses in that they attack only those cells which have the necessary enzymes or some other specific mechanism.

Plant Viruses.—Entirely different results were obtained when the smaller viruses were studied. Stanley (1935) isolated the virus of tobacco mosaic from infected plants and prepared the agent in a highly purified crystalline form. This preparation was found to consist entirely of protein. Repeated recrystallizations failed to change the infectivity of the crystals. The protein could be denatured or changed in other ways resulting in a partial or complete loss of infectivity. Analysis of the crystals showed that they were similar in composition to other proteins except that the phosphorus content was relatively low. Molecular weight determinations of the crystals by several methods gave a value of about 17,000,000, a tremendous figure for a protein. More recently Frampton and Saum (1939) by another procedure obtained a molecular weight figure of only 100,000.

Antigenicity of Plant Viruses.—The tobacco mosaic virus crystals were found to be antigenic when injected into rabbits, resulting in the elaboration of specific precipitins. The immune serum gave some cross reaction with the protein normally present in the healthy tobacco plant. This observation is in support of the theory of Stanley, which states that the virus is a polymer of the protein normally present in the tobacco plant and that its formation is the result of the autocatalytic activity of a small amount of the polymer.

Bawden and Pirie (1937) found that Stanley's virus crystals contained small amounts of nucleic acid and that its removal destroyed the infectivity of the crystals. Others have confirmed the findings of Bawden and Pirie.

Resistance of Viruses.—Viruses are, in general, more resistant to the action of unfavorable conditions than are the bacteria. The addition of 0.5 per cent phenol to vaccine virus does not destroy its infectivity. Glycerin, in a concentration of 50 per cent, gradually destroys bacteria but has no appreciable effect on viruses. On the other hand viruses are very susceptible to oxidative destruction. This is largely prevented by the addition of a suitable reducing substance, such as cysteine, to the virus suspension.

TABLE 49.—VIRUS DISEASES WITH CELL INCLUSIONS WHICH ARE DEFINITELY PROVED TRANSMISSIBLE AND IN WHICH FILTERABILITY OF THE CAUSATIVE AGENT IS ESTABLISHED

A. Diseases of Man

Virus	Brief characterization of the disease
1. Smallpox: *a.* varioloid *b.* vaccinia *c.* paravaccinia *d.* alastrim	Smallpox is an acute, specific, infectious disease characterized by sudden onset, usually with severe chill, with rapidly rising temperature, followed by an eruption passing through papular, vesicular, and pustular stages. Permanent scars frequently remain Varioloid, vaccinia, paravaccinia, and alastrim are milder forms of the disease, presumably caused by the same virus, which has become altered in virulence
2. Rabies (hydrophobia, canine madness)	Infection follows the bite of an infected animal, usually the dog. Disease characterized by depression, itching at sight of primary infection, and fever. Patient becomes uneasy, swallowing difficult, salivation marked, followed by attacks of delirium. Paralysis of the face muscles, eyes, and tongue appears, gradually spreading to the trunk and limbs
3. *Herpes simplex* (fever blisters, cold sores)	*Herpes simplex* is an acute eruptive disease of the skin and mucous surfaces, usually occurring on the face and genitalia. The eruption is vesicular and the vesicles occur in groups accompanied by a mild inflammation and a sensation of burning
4. Yellow fever (*typhus icteroides*)	Yellow fever is an acute, specific disease accompanied by fever, chills, muscular pain, some destruction of red blood cells, black vomit, albumin in the urine, and jaundice. The disease is transmitted to man by the bite of the mosquito *Aedes aegypti*
5. Psittacosis (parrot fever)	Psittacosis is a contagious disease of parrots resembling influenza and transmissible to man. In man the disease is characterized by high fever, headache, backache, thirst, changes in the tongue and pharynx, stupor or depression, fast pulse, diarrhea or constipation, swelling of the spleen, symptoms of an atypical pneumonia or of a typhoidal state, with *râles* and cardiac dullness, and the usual external signs of catarrh and pneumonia may be absent

TABLE 49.—(*Continued*)
B. Diseases of Lower Animals

Virus	Brief characterization of the disease
1. Pox diseases: *a.* cowpox *b.* sheeppox	Cowpox, or vaccinia, is an acute infectious disease of cattle characterized by fever and a vesicular exanthema. The disease is closely related to smallpox in man Sheeppox is an acute infectious disease of sheep characterized by fever and a papulovesiculopustular eruption accompanied by catarrh of the respiratory passages
2. Foot-and-mouth disease	Foot-and-mouth disease is an acute, contagious disease of cloven-footed animals. The disease is characterized by a vesicular eruption on the mucous membranes and skin
3. Rabies (hydrophobia, canine madness)	Rabies is characterized as an acute, highly specific infection of the central nervous system. It is primarily a disease of animals. Disease transmitted from animal to animal and from animal to man
4. Hog cholera (swine fever, typhoid fever)	Hog cholera is an acute, septicemic disease of swine characterized by an acute febrile reaction accompanied by an inflammatory swelling of the conjunctiva, eczematous eruption of the skin, and diarrhea

VIRUS DISEASES WITH CELL INCLUSIONS WHICH ARE TRANSMISSIBLE BUT IN WHICH FILTERABILITY OF THE CAUSATIVE AGENT HAS NOT BEEN ESTABLISHED
Diseases of Man

1. Varicella (chickenpox)	Varicella is an extremely contagious disease characterized by a cutaneous eruption involving the superficial layers of the skin

VIRUS DISEASES WITH CELL INCLUSIONS WHICH ARE NOT TRANSMISSIBLE AND IN WHICH FILTERABILITY OF THE CAUSATIVE AGENT HAS NOT BEEN ESTABLISHED
Diseases of Man

1. Herpes zoster (shingles)	Herpes zoster is an acute disease characterized by the appearance of vesicles that follow the course of a nerve trunk and accompanied by pain or itching

TABLE 49.—(*Continued*)

VIRUS DISEASES WITH NO CELL INCLUSIONS WHICH ARE TRANSMISSIBLE AND IN WHICH THE FILTERABILITY OF THE CAUSATIVE AGENT HAS BEEN ESTABLISHED

A. Diseases of Man

Virus	Brief characterization of the disease
1. Epidemic parotitis (mumps)	Epidemic parotitis is a specific contagious disease characterized by inflammation of the salivary glands. The parotid, the submaxillary, and the sublingual glands may be infected, although the parotid is most frequently involved. Sometimes the ovaries and testes may be attacked. The disease is transmissible from person to person and to experimental animals
2. Acute anterior poliomyelitis (infantile paralysis)	Poliomyelitis is an acute, systemic, infectious disease that involves the central nervous system
3. Common colds (acute coryzas)	Common colds are acute, exudative, and catarrhal infections of the nose, throat, larynx, sinuses, trachea, and larger bronchi

B. Diseases of Lower Animals

1. Rinderpest (cattle plague)	Rinderpest is an acute, contagious disease of cattle characterized by fever, formation of a fine pseudomembrane upon the mucous surfaces, increased salivation, and usually a nasal discharge with profuse bloody diarrhea
2 Pleuropneumonia of cattle (lung plague)	Pleuropneumonia is an acute, subacute, or chronic disease of cattle characterized by an exudative inflammation of the interlobular lymph vessels, and of the alveolar tissue of the lungs, accompanied by a serofibrinous pleurisy

DISEASES OF UNKNOWN OR QUESTIONABLE ORIGIN FOR WHICH SPECIFIC AGENTS HAVE BEEN CLAIMED. INCLUSION BODIES HAVE IN CERTAIN INSTANCES BEEN REPORTED

A. Diseases of Man

1. Scarlet fever	Scarlet fever was at one time reported to be a virus disease but the etiological agent has now been shown to be a streptococcus (see p. 583)
2. Epidemic encephalitis (*encephalitis lethargica*)	Epidemic encephalitis is a disease of the central nervous system and is characterized by inflammation, lethargy, paralysis of the cranial nerves, and in some cases spinal and peripheral nerve involvement. Fever occurs during the acute stage or on each exacerbation

TABLE 49.—(*Continued*)

Virus	Brief characterization of the disease
3. Epidemic influenza (la grippe)	Epidemic influenza was formerly believed to be due to the organism *Hemophilus influenzae*, but has now been shown to be caused by a filterable virus. In typical, severe cases *Hemophilus influenzae* is also present but is probably of secondary importance (see page 574) Influenza is an acute infection characterized by fever, catarrh of the respiratory tract (sometimes alimentary tract), pains in the head and muscles, and a tendency to pneumonic complications. The disease appears suddenly and produces extreme prostration
4. Measles (rubeola, morbilli)	The cause of measles is still undetermined but is probably due to a filterable virus. It is a specific, highly contagious disease characterized by fever, catarrh of the upper respiratory passages, suffusion of the eyes, and an accompanying, cutaneous rash
5. German measles (rubella)	German measles is a specific, mild, infectious disease characterized by a cutaneous eruption, which usually appears without other symptoms. The cause is still undetermined but is believed to be due to a filterable virus
6. Psoriasis	Psoriasis is a chronic inflammatory disease characterized by dry, reddish patches, which usually appear on the extensor surfaces and are covered with whitish or grayish silvery scales. The cause of the disease is still undetermined but is believed by some to be due to a filterable virus
	B. Diseases of Lower Animals
1. Swine influenza	Swine influenza is caused by the associated activities of two organisms: (1) an influenza-like bacillus and (2) a filterable virus Animals receiving injections of the bacilli alone remain normal; animals receiving virus alone develop a mild disease; animals receiving both bacilli and virus develop a typical swine influenza

Immunity in Virus Diseases.—Animals that have recovered from virus infections usually exhibit a certain amount of immunity to a subsequent infection with the same virus. Various kinds of immune substances have been identified in the circulating blood, including agglutinins, precipitins, and neutralizing antibodies. In some virus diseases

the immunity may persist throughout life, whereas in others it may be of short duration. An example of the former is smallpox; an example of the latter is *herpes simplex* (cold sores, fever blisters) where frequent recurrence is almost the rule.

Recovery from some virus diseases does not mean that the etiological agent is entirely eliminated from the host. The virus may persist in the blood stream and continue to multiply without showing any special affinities for cellular tissues. For example, horses are susceptible to the virus disease known as infectious equine anemia. Horses that have recovered from the disease are immune from further attack. The horses continue to carry the virus in their blood for the remainder of their lives and thus act as virus reservoirs. The injection of blood from a horse that has recovered from the disease into a normal healthy horse results in an infection.

A classification of some important viruses pathogenic for man and animals based on the presence or absence of inclusion bodies, according to McKinley (1935), is given in Table 49.

For further information on the viruses consult the monographs and articles by Barnard (1935), Bawden and Pirie (1938), Bedson (1937), Dale (1935), Doerr and Hallauer (1938), Findlay (1939), Gildemeister, Haagen, and Waldmann (1939), Harvard School of Public Health Symposium (1940), Hurst (1936), Laidlaw (1938), Levaditi (1938), Levaditi and Lépine (1938), Rivers (1936, 1937, 1938), and Soule (1940).

RICKETTSIAL DISEASES

The rickettsial diseases of man may be defined as specific infections transmitted by the bite of arthropods and characterized by continued fever, accompanied by a rash. The pathological lesions occur chiefly in the blood vessels, being due to the presence of the organisms and not to their toxins.

Rickettsial Organisms.—The rickettsial organisms are so named in honor of Howard Taylor Ricketts. Ricketts (1909) was the first to give a description of the organisms in connection with his studies on Rocky Mountain spotted fever and later on typhus fever. He succeeded in isolating from the blood of typhus fever patients very short bacillus-like rods measuring about 0.3μ in diameter and 2μ or less in length. The organisms were stained readily by Giemsa's stain and possessed a faintly stained bar through the middle, giving each organism the appearance of a diplobacillus. The organisms were nonmotile. The observations of Ricketts were later confirmed by da Rocha-Lima (1916).

Following the discovery of Ricketts, other workers, notably Hegler and Prowazek (1913), reported the presence of similar organisms in the blood of typhus fever patients and in lice that had fed on infected indi-

viduals. The organisms were described as bipolar bodies or paired granules consisting of faintly staining substance taking a heavy stain at the poles by the method of Giemsa.

The two most important organisms causing rickettsial diseases are *Rickettsia prowazeki*, the cause of typhus fever, and *R. dermacentroxenus*, the cause of Rocky Mountain spotted fever. Other rickettsias that have been shown to cause disease include; *R. wolhynica*, *R. quintana*, and *R. pediculi*, the causative organisms of trench fever; *R. tsutsugamushi*, the cause of tsutsugamushi disease; and *R. ruminantium*, the organism responsible for heartwater disease.

ROCKY MOUNTAIN SPOTTED FEVER

Rocky Mountain spotted fever is a specific infectious disease characterized by a macular eruption, accompanied by irritability and hyperesthesia of the skin, and enlarged spleen and catarrh of the respiratory tract. Sometimes albumin and casts are found in the urine. The disease is caused by the organism *Rickettsia dermacentroxenus* and occurs all over the United States. In the west the organism is carried by the wood tick *Dermacentor andersoni*, and in the east by the dog tick *D. variabilis*. The organism is also pathogenic for rabbits, guinea pigs, ground squirrels, woodchucks, and monkeys.

The disease is transmitted to man by the bite of the adult tick, but the larvae and nymphs may transmit the disease to lower animals. The organisms occur in the circulating blood and in the endothelial cells of the testes. The rods invade all the tissues of the tick. In man the organisms are found chiefly in the smaller peripheral blood vessels where they occur in pairs or in clusters, and cause a proliferation of the endothelium of the arteries and veins of the skin, subcutaneous tissues, and testes.

Recovery from an attack of the disease usually confers lasting immunity. The blood serum from recovered cases confers protection to guinea pigs. A vaccine prepared by emulsifying infected ticks in a 5 per cent solution of phenol is capable of immunizing man and animals against the disease.

TYPHUS FEVER

Typhus fever is an acute infectious disease characterized by a rash, causing in most cases a lethargic or typhus state, accompanied by nausea, headache, and dizziness. Sometimes convulsions may occur and the patient may pass into a delirium. The causative agent of the disease is *Rickettsia prowazeki*. It is transmitted to man by the bite of the rat flea *Xenopsylla cheopis* and probably by other fleas; from man to

man by the body louse *Pediculus humanus;* from rat to rat by the rat flea *X. cheopis* and the rat louse *Polyplax spinulosus.*

R. prowazeki is a small rod measuring 0.25 to 0.3 by 0.3 to 1.1μ. It is found in the circulating blood of typhus fever patients. The organisms occur in pairs and in clusters and are readily stained by Giemsa's method. The rods are pathogenic for guinea pigs. The organisms do not multiply on laboratory media but have been successfully cultivated in tissue cultures. They do not pass through Berkefeld filters. In human infections the organisms may be found in the blood vessels of the skin, kidneys, muscles, brain, and testes. The incubation period of the disease in man is from 4 to 14 days.

The most important lesions include a perivascular infiltration of cells followed by a proliferation and disintegration of the endothelium, resulting in the formation of hemorrhages and thrombi. The skin lesions give rise to the characteristic bright red spots. Similar lesions are found in the blood vessels of the kidneys, brain, and testes.

Recovery from an attack confers a solid immunity but is not always permanent. Second and third attacks have been known to occur. Serum from typhus fever patients agglutinates certain strains of *Proteus* which are designated *Proteus* $X2$ and *Proteus* $X19$. The reaction is very important from a diagnostic standpoint. A vaccine prepared by emulsifying infected ticks in a 5 per cent solution of phenol is capable of producing an active immunity in man and animals.

TSUTSUGAMUSHI DISEASE

Tsutsugamushi disease is a specific infection of man prevalent in Japan and is characterized by fever, chills, swelling and tenderness of the lymph glands, and an accompanying skin eruption. The spleen and liver are congested and there may be cloudy swelling in the parenchymatous organs and cellular necrosis. The insect vector is the larva of the mite known as *Trombicula akamushi.* Primary sores develop at the site of the insect bite, caused by the secretions of the insect and accompanied by adenitis of the regional lymph nodes. The disease is caused by the organism *Rickettsia tsutsugamushi* (*R. orientalis*, *R. akamushi*). It is not known if the infectious agent is a virus or a visible organism. The etiological agent is capable of passing through Berkefeld filters.

Recovery from an attack of the disease confers some immunity but second attacks do occur. The serum of a patient fails to agglutinate *Proteus* $X2$ and *Proteus* $X19$ but gives positive results with *Proteus* XK.

For further information on the rickettsial diseases consult the monographs and articles by Biraud and Deutschman (1936), Cox (1938), Dyer (1935, 1938), FitzPatrick (1938), Hampton and Eubank (1938), Harvard School of Public Health Symposium (1940), Parker (1937, 1938),

Pinkerton (1936), Zinsser (1937), and Zinsser, FitzPatrick, and Wei (1939).

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CHAPTER XXVII

THE HISTORY OF BACTERIOLOGY

The history of disease dates back to biblical times, but it has been comparatively recently that bacteria were first seen and recognized as the causative agents of infections.

Benedetti (1493) stated that disease was contracted by touch and that the causative agent was taken up by various articles used by the patient. Fracastoro (1483–1553) postulated the existence of seeds of disease, which he named *seminaria prima*. These seeds were capable of being disseminated from person to person. He believed that the seeds were transmissible (1) by direct contact, (2) by contact and fomites, and (3) at a distance. The term fomes (pleural, fomites) may be defined as any substance, such as clothing, capable of absorbing, retaining, and transporting infectious organisms. The seeds of infection were believed by him to invade and multiply until the entire mass was involved. Fracastoro accurately described the great epidemics of typhus fever, plague, rabies, and syphilis that occurred in Italy from 1505 to 1528. It is not known whether he considered the seeds of infection as living or dead agents.

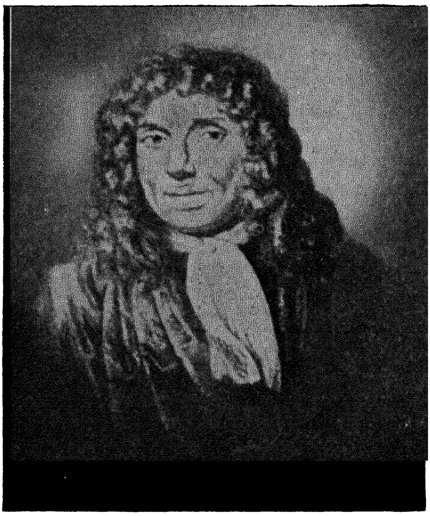

FIG. 239.—Antony van Leeuwenhoek.

ANTONY VAN LEEUWENHOEK

The discovery of living microscopic organisms is generally accredited to the Dutch microscopist Antony van Leeuwenhoek (circa 1672). He was not a trained scientist but a skilled craftsman in glass blowing, metal working, lens grinding, etc. Leeuwenhoek amused himself by grinding lenses and making his own microscopes, which were nothing more than simple magnifying glasses. The magnifying powers of some 400 microscopes that he made ranged from 40 to 300 diameters. A sketch

of one of his microscopes is shown in Fig. 240. Leeuwenhoek examined rain water, well water, infusions of peppercorns, tartar scraped from teeth, throat washings, etc. His lenses are not to be compared to the modern high-power objectives but the pictures that he sketched in his letters to the Royal Society of London leave no doubt that he actually saw living microscopic organisms (Fig. 241). Although van Leeuwenhoek is generally referred to as the first bacteriologist and protozoologist, he was not the first individual to make or use a microscope.

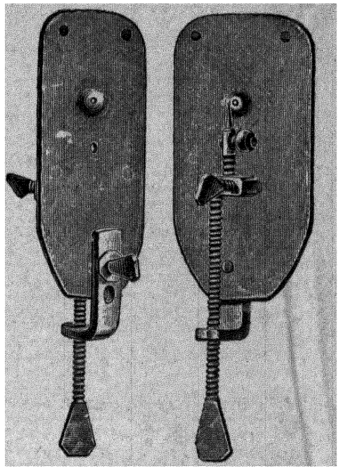

FIG. 240.—One of van Leeuwenhoek's microscopes (ante 1673). (*From "Origin and Development of the Microscope," courtesy of Royal Microscopic Society, London.*)

Compound Microscopes.—Compound microscopes were in use many years before the epoch-making observations of van Leeuwenhoek. Pieter Harting (1866) believed that the compound microscope was first invented by Zacharias Janssen (about 1590).

Living Organisms as Cause of Disease.—The observations of van Leeuwenhoek were instrumental in reviving the ancient belief, held by

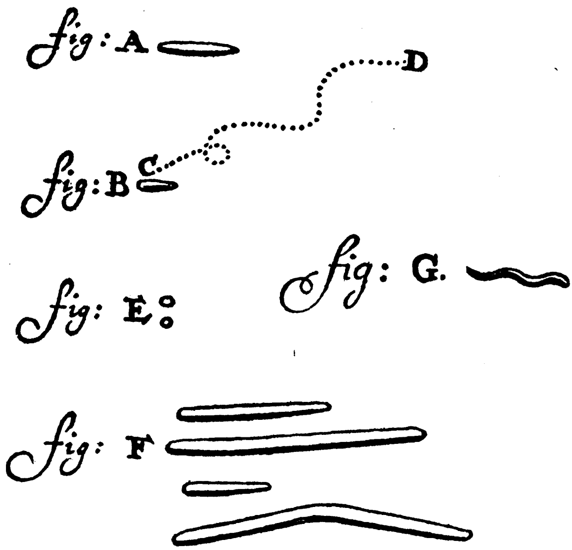

FIG. 241.—Leeuwenhoek's drawings of bacteria. These are the oldest known figures of bacteria.

Varro (116–26 B.C.), Columella (about 60 B.C.), and others, that diseases might be caused by living microscopic organisms. Some of those who

believed that diseases were caused by living organisms included Kircher (1658), Lange (1659, 1688), and Lancisi (1718). During this same period Marten (1720) expressed his views concerning the etiological agent of tuberculosis, which coincided exactly with the ideas held by present-day investigators. Since he could not offer any support for his statements, many doubted his views. Plenciz (1762) was perhaps the first to state that the seeds of contagion were air-borne and capable of developing into innumerable organisms.

Fig. 242.—Zacharias Janssen, inventor of the compound microscope.

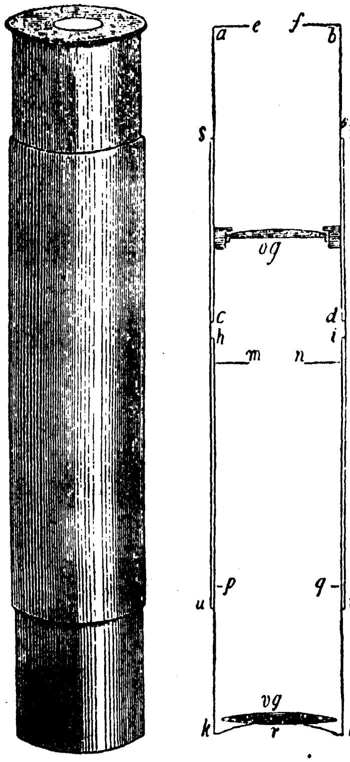

Fig. 243.—The Middelburg microscope, believed to be the invention of Zacharias Janssen (1590). (*From "Origin and Development of the Microscope," courtesy of the Royal Microscopic Society, London.*)

FERMENTATION

Industrial fermentations such as occur in the preparation of wines, bread, vinegar, beer, etc., were widely practiced by the ancients, but they were little understood. Gay-Lussac (1810) believed that oxygen was necessary to start fermentation, but once started, the gas was no longer required.

During the early part of the nineteenth century the yeast plant was discovered and offered a new method of approach to the phenomenon of fermentation. Cagniard-Latour (1836) was probably the first to

express the belief that yeast was a living plant. The following year he stated that fermentation was probably the result of the growth and multiplication of yeast cells in sugar solutions with the production of alcohol and carbon dioxide. He even noted that yeast cells multiplied by the process of budding. Schwann (1837), working independently of Cagniard-Latour, also expressed the belief that yeast was a plant. He stated that, in addition to sugar, a nitrogenous compound was necessary for the growth of the organisms. The plants utilized the sugar and nitrogen compound and eliminated alcohol and carbon dioxide as waste products. His reasons for believing that yeasts caused fermentation were (1) They were always found to be present during the fermentation process; (2) the destruction of the cells resulted in a cessation of the fermentation process; and (3) the substance causing fermentation (enzymes) was regenerated and increased in amount during the process. This latter phenomenon is characteristic only of living bodies.

Unfortunately, the biological theory of fermentation made slow progress, owing to the fact that the majority of scientists of that period were chemists and physicists. Berzelius (1779–1848), and his pupils Wöhler (1800–1882) and Liebig (1803–1873), were chemists imbued with the belief that all vital phenomena could be explained on purely chemical grounds. These three chemists vigorously opposed the biological theory by ridiculing the teachings of Schwann and Cagniard Latour in every possible manner. Liebig believed that fermentation and putrefaction were the result of the chemical instability of certain substances that were capable of transferring their instability to other substances. He called these unstable bodies ferments. The ferments were believed to arise from some constituent of the solution when exposed to air, but after fermentation had started, oxygen was no longer required.

Mitscherlich (1841) showed that yeast cells were not able to pass through the pores of filter paper. He wrapped a piece of filter paper around one end of a short length of glass tubing and suspended it in a jar of sugar solution. The inside of the tube was filled with the same solution to which had been added a few yeast cells. Fermentation occurred inside of the tube but not in the surrounding solution. Although the experiment was convincing for the biological theory of fermentation, Mitscherlich explained the result by stating that yeast did not act as a vital agent, but merely by its presence. He adhered to the chemical teachings of Berzelius, Wöhler, and Liebig.

Louis Pasteur.—The early discoveries in the field of fermentation were made by Pasteur. About 1854 Pasteur interested himself in this field and shortly thereafter published his first report on the fermentation of sugar to lactic acid. He noted a grayish deposit and scum in liquids undergoing the lactic fermentation. When some of the material from

the sediment and scum was examined under the microscope, it was found to be composed of a mass of minute round bodies. Transfer of some of these bodies to a fresh solution of sugar resulted in a lactic fermentation. This process could be repeated indefinitely. He stated that the spherical cells were living organisms and that they were responsible for the fermentation. When sterilized solutions of sugar were exposed to the air the same type of fermentation was again produced. Examination of the scum and sediment revealed the same globoid bodies. He argued that the yeast cells came from the air because, when sterilized solutions were protected from the air, no fermentation occurred and no yeast cells appeared in the solution.

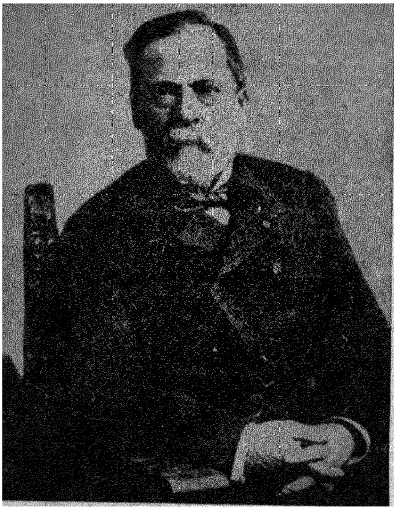

FIG. 244.—Louis Pasteur.

During the next 20 years Pasteur devoted most of his time to a study of all types of fermentations. He showed that each type was produced by a specific organism and that in the absence of living cells no fermentation occurred. He described various "diseases of wines," which were caused by contaminating or foreign organisms, resulting in abnormal fermentation.

Pasteur devoted the period from 1865 to 1871 to a study of the silkworm disease, which was ravaging France at that time. After he had completed his memorable work on this disease, he again returned to a study of the various types of fermentations. He expressed the view that all fermentations were anaerobic reactions, that they occurred in the absence of free atmospheric oxygen.

Buchner (1896) subjected yeast cells to great pressure and collected the cell-free juice. He found that this liquid, when added to a solution of sugar, was capable of inducing alcoholic fermentation. This observation brought to an end the controversies of Pasteur vs. Berzelius, Wöhler, and Liebig. It showed that both views were correct in that fermentation took place in the absence of living cells by means of enzymes, but that a living organism was necessary to elaborate the enzymes (ferments).

SPONTANEOUS GENERATION

The ancients believed that living organisms were created through the combined action of heat, earth, air, and putrefaction. Several of the more ardent supporters of the theory even went so far as to offer recipes

for spontaneous generation. The most famous of these was the one advocated by van Helmont (1652). He stated that if a piece of dirty linen cloth were stuffed into the neck of a jar containing grains of corn, a fermentation ensued and after a lapse of 21 days mice appeared. What surprised van Helmont was that the mice that appeared were always of adult size and fully formed.

For the next 200 years scientists and philosophers were concerned with the problems of spontaneous generation, some supporting the theory and others vigorously opposing it. Redi (1668) expressed the view that maggots found in putrid meat were the offspring of flies. He covered pieces of meat and fish with fine-mesh gauze and placed them under gauze-covered supports. Flies were allowed to come in contact with all parts of the gauze, but no maggots developed. He believed that he actually saw flies deposit their eggs on the gauze. Redi also showed that, when maggots were kept in a closed vessel, flies developed. Convincing as these experiments were, the idea of spontaneous generation still persisted.

Leeuwenhoek, the discoverer of bacteria, believed that the animalcules that appeared in infusions came from the air, where they were present as seeds and developed into active organisms after they gained entrance into infusions. Joblot (1718) boiled hay infusion and placed equal quantities into two jars. One was covered with parchment while still hot; the other was exposed to the air. The open jar was soon teeming with bacteria; the covered jar remained sterile. When the closed jar was exposed to the air, it soon became heavily contaminated. These observations undoubtedly refuted the doctrine of spontaneous generation but there were still some who did not regard them as conclusive.

During the next few years a strong controversy developed between Needham, a proponent of the theory of spontaneous generation, and Spallanzani. Needham (1749) believed that in every particle of organic matter there existed a vegetative force that caused the organic matter of the medium to be converted into living forms. Spallanzani (1765) carried out hundreds of experiments to refute the teachings of Needham. He heated infusions in various kinds of flasks, after which they were stoppered with cork, wood, or paper. He was not certain that he had excluded all air that was believed to act as a carrier of the seeds. Some of his infusions were boiled after all air had been removed, then distributed into sterile flasks aseptically and hermetically sealed. He concluded not only that the infusion must be sterile, but that the air must be free from living organisms. If both are sterile and the containers properly sealed, organisms will not develop in the medium. Needham criticized the experiments by stating that the prolonged heating had destroyed the vegetative force of the liquid. Spallanzani continued to

answer every objection put forth by Needham, but still he was not able to convince the proponents of the doctrine of spontaneous generation.

Schulze (1836) devised an experiment (Fig. 245) in which the infusion was sterilized by heat and the overlying air freed from living organisms by filtration. The flask containing the medium was boiled over a sand bath and, while steam was streaming from the vessel, he attached an absorption bulb at each end. One bulb contained sulfuric acid while the other held a solution of potassium hydroxide. Schulze applied his mouth

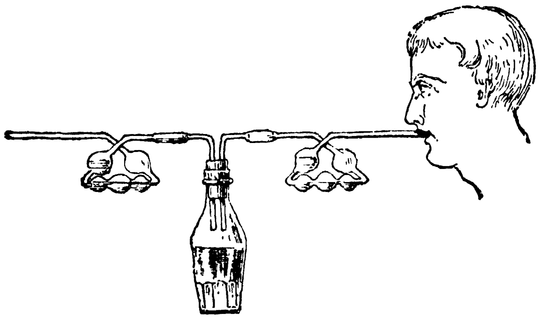

FIG. 245.—Schulze's experiment (1836). (*After Lafar.*)

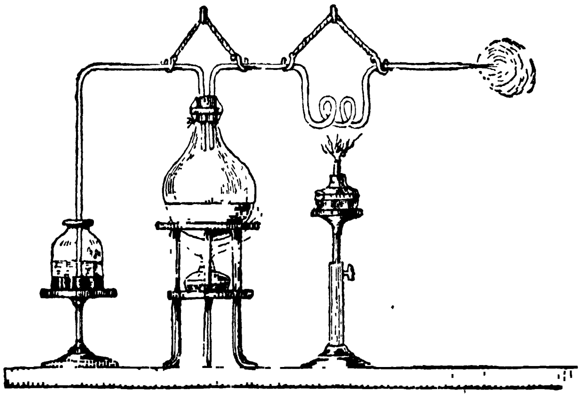

FIG. 246.—Schwann's experiment. (*After Lafar.*)

to the open end of the potash bulb and aspirated fresh air into the vessel containing the medium. The air, in passing through the sulfuric acid, was freed from bacteria. The medium remained sterile for more than two months. A control flask showed growth in two days. The experiment demonstrated that the air in the flask was not the cause of growth in the liquid. Schwann (1837) performed a similar experiment (Fig. 246) in which he did away with the absorption bulbs. One tube from the flask entered a vessel containing mercury; the other opening was connected to a piece of glass tubing having several spiral twists. The infusion was brought to a boil and, while the steam was streaming from

the open end, heat was also applied to the spiral. When the contents of the flask had cooled, the open spiral end was sealed in a flame. The contents of the flask remained sterile for six weeks. When the flask was disconnected putrefaction rapidly took place. Helmholtz (1843), in a similar type of experiment, confirmed Schwann's results.

Schröder and von Dusch (1854) filtered air through cotton instead of heating or drawing it through sulfuric acid. Later Schröder (1859) stoppered flasks with cotton. Solutions boiled in plugged flasks remained

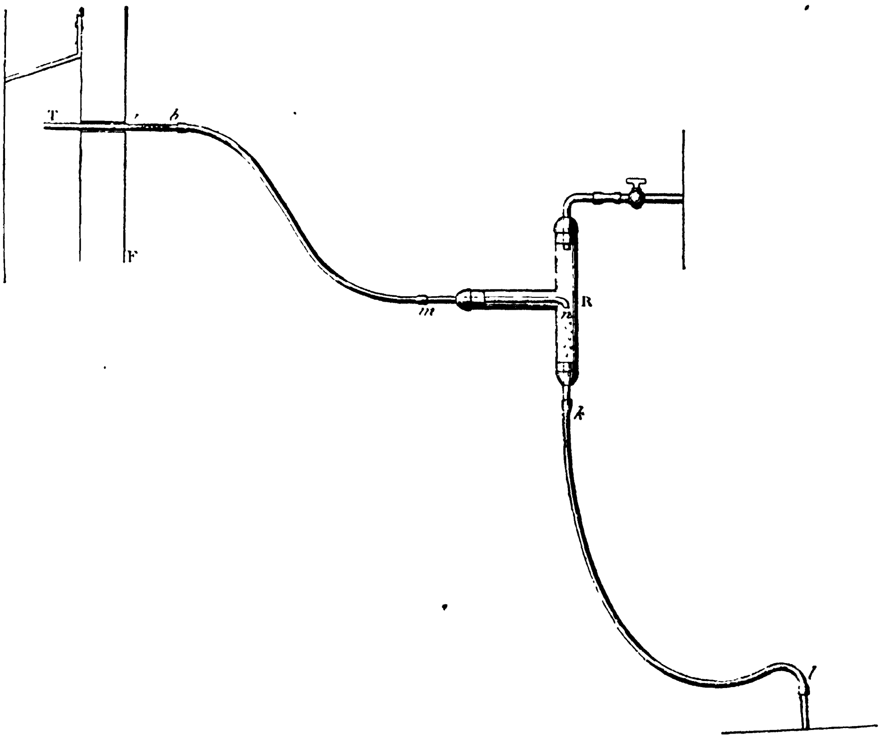

FIG. 247.—Experiment of Pasteur. Aspirated air filtered through a pledget of gun cotton (*a-b*).

sterile, demonstrating that the cotton prevented bacterial organisms from entering the flasks. This was probably the origin of the use of cotton stoppers for keeping laboratory glassware and culture media sterile. Convincing as these experiments were, they still did not convert the proponents of the doctrine of spontaneous generation.

About this time Pouchet came into prominence by bitterly opposing the teachings of Pasteur and Spallanzani. He performed many experiments that convinced him that living organisms are able to spring from dead organic matter. The experiments of Schwann and Schulze were repeated by him with opposite results. Pasteur devoted the next several

years devising experiments to refute the teachings of Pouchet. The apparatus used in one of these experiments is shown in Fig. 247. The aspirated air was filtered through a pledget of gun cotton ($a - b$). At the end of the experiment the gun cotton was removed from the tube and dissolved in a mixture of ether and alcohol. The sediment that settled out at the end of 24 hr. was examined under the microscope and found to consist of many organisms (Fig. 248). He also filled flasks with infusion and bent the necks downward in such a manner that organisms from the air could not ascend the tube and enter the flask (Fig. 249). The infusion was then boiled to destroy any living organisms present. The flasks remained sterile for months. When the necks were cut off, putrefaction rapidly set in. Pasteur showed that the number of organisms present in air varied with the locality. He exposed 20 flasks at an altitude of

FIG. 248.—Air particles collected on the piece of gun cotton (a-b) in Pasteur's experiment (Fig. 247).

2550 ft. and 20 at 6276 ft. At the end of the experiment the former group showed 12 flasks sterile and 8 contaminated, whereas the latter showed 19 sterile and only 1 contaminated. These experiments again demonstrated that bacteria were present in air and also that the numbers present decreased with altitude. Still Pouchet refused to be shaken by Pasteur's latest experiments.

Pouchet exposed flasks at an elevation of 9000 ft. and much to his satisfaction all of them became turbid. He obtained air from the summits of Mont Blanc and Monte Rosa and found that organisms were present. His experiments gave results just the opposite from those obtained by Pasteur. It was finally arranged to have Pasteur and Pouchet appear at Chevreul's laboratory in the Museum of Natural History. Pasteur performed a number of convincing experiments before a commission appointed to decide the controversy but Pouchet refused to carry out any experiments. This was sufficient to convince the leading scientists of the day that the conclusions of Pasteur were correct. Pouchet finally gave up the problem and dropped from public view.

Bastian (1872) continued to propound the beliefs of Pouchet for nearly 40 years, during which time he published many books and papers on the subject. In the end he appeared to be alone in his fight for the establishment of the doctrine of heterogenesis.

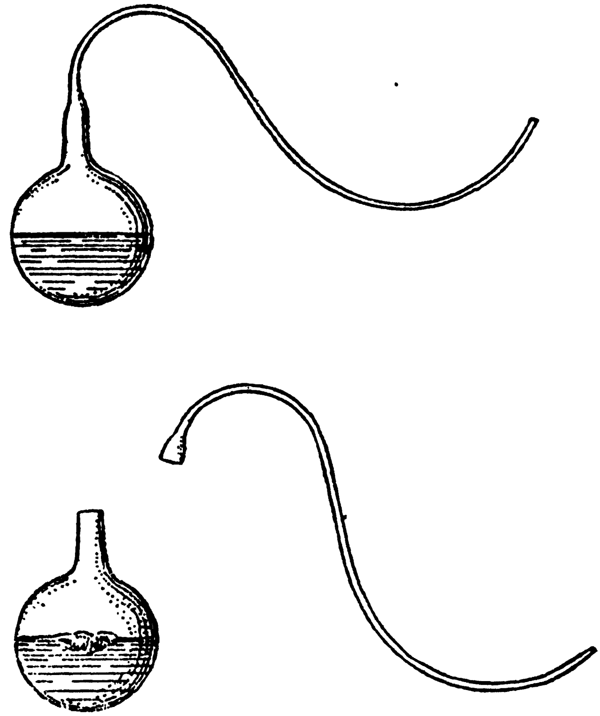

FIG. 249.—Long-neck flasks used by Pasteur to prevent entrance of dust to the interior.

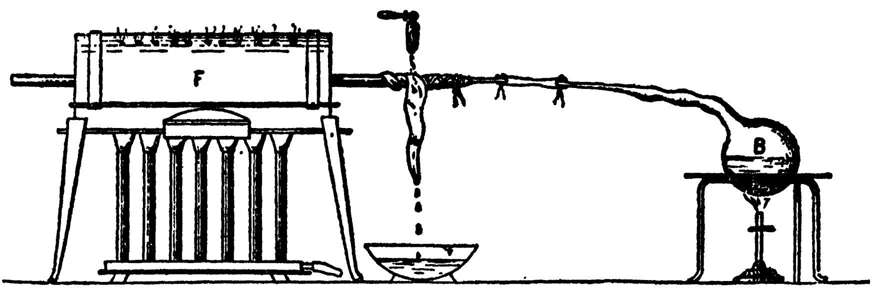

FIG. 250.—One of Pasteur's experiments to refute the doctrine of spontaneous generation.

Tyndall (1876) noticed that, when a beam of light was passed through air laden with dust particles, there was a scattering of the light ray into its spectrum. If the dust particles ~~were absent,~~ no scattering effect was noted. After this observation he employed a beam of light to reveal the presence of dust particles. Tyndall devised a special chamber

(Fig. 251) to study the development of organisms in culture media. Two small holes in the sides and the entire front were covered with glass. A hole in the top was covered with a piece of sheet rubber, in the center of which was inserted a long thistle tube, *p*. Two bent tubes were inserted into openings *a* and *b*. The tubes were bent several times to prevent the entrance of any particles into the chamber. The floor was perforated with holes to accommodate 12 large test tubes. The interior surfaces of the chamber were moistened with glycerin to gather the

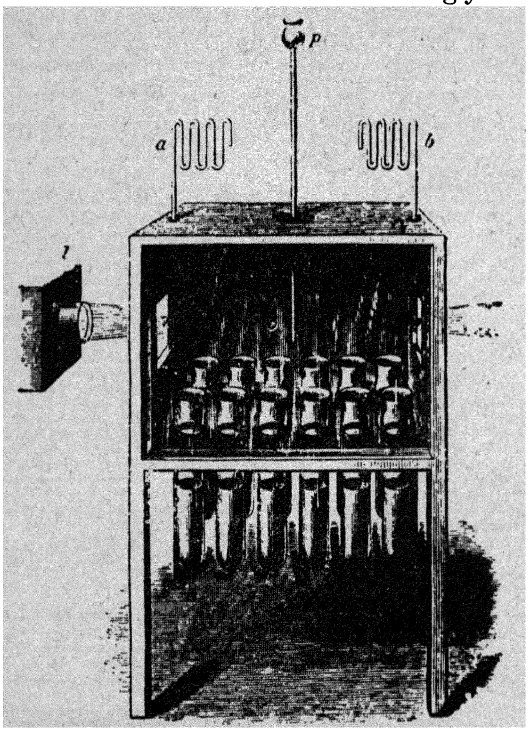

Fig. 251.—Tyndall's chamber for experiments on spontaneous generation.

floating dust particles coming in contact with the liquid. The chamber was allowed to stand undisturbed for several days until a beam of light showed the interior to be free from floating dust particles. Then the tubes were filled with culture media through the thistle tube. Since this tube was held in the flexible sheet of rubber, the stem could be easily directed into each test tube. The tubes were finally immersed in a boiling brine bath and held at that temperature for 5 min. The protected tubes of medium in the chamber remained sterile for months, and the control tubes exposed to the air showed growth after a few hours. He concluded that the contamination of the medium and the scattering of the light rays by the dust particles ran parallel.

Tyndall noted that sometimes 5-min. heating of the tubes in a boiling brine bath did not always sterilize the medium. He believed that some organisms were relatively thermolabile and destroyed in 5 min. at 100°C. and that others were thermoresistant to a high degree. Cohn (1876) arrived at the same conclusion independently of Tyndall by different methods. Tyndall showed that if the medium was boiled for 1 min. on five successive days it remained sterile. This appeared to be the first reference to use of the discontinuous method (Arnold) for the sterilization of culture media.

Cohn (1876) showed that the thermoresistance of some organisms was due to the presence of certain highly refractile bodies known as spores. Old cultures of hay infusion revealed many spores, whereas fresh cultures showed a much smaller number. He stated that when spores were transferred to a fresh medium, germination into vegetative cells occurred. Cohn is generally given credit for being the first observer to see and describe spores of bacteria.

PUTREFACTION

For centuries putrefaction was considered an important factor in the production of diseases. Complications, known as sepsis, putrid intoxication, etc., were believed to be the result of the absorption of putrid substances into the system. This belief was the outcome of observations on the injection of putrid materials into animals, which resulted in the development of symptoms resembling those noted in septic infections. Gaspard (1822) conducted many experiments on animals and birds by injecting them with putrid substances, and described in great detail the symptoms and lesions that followed. Gaspard's work was repeated by others and aroused great interest at the time.

Magendie (1823) was the first to show that a dose of putrid material was lethal if injected intravenously, but inert if given by mouth. He placed animals on a grill above a putrid mass of material and found that no ill effect resulted from the inhalation of the foul gases given off. This observation refuted the ancient belief that disease was caused by the penetration of foul odors into the respiratory tract.

Schwann (1837) suggested and actually demonstrated that putrefaction was a biological process. This observation was later confirmed by Pasteur (1863), who stated that putrefaction was due to "ferments" of the genus *Vibrio*. Following the report of Pasteur some workers attempted to show that putrefaction was due to chemical poisons while others believed it was due to the growth of living organisms. Later Brieger (1881) isolated a large number of compounds known as ptomaines and believed to be the cause of disease. At present it is definitely established that ptomaines are not capable of producing disease.

PYEMIA AND SEPTICEMIA

Piorry (1837) believed that certain infections, leading to the formation of abscesses in various parts of the body, resulted in an escape of pus into the blood stream. He called this condition pyemia. Pyemia may be defined as a form of blood poisoning produced by the absorption of pyogenic microorganisms into the blood, usually from a wound or local inflammation. It is characterized by multiple abscesses throughout the body. Septicemia may be defined as a poisoned condition of the blood due to the presence of pathogenic bacteria. It is sometimes referred to as blood poisoning. Donné (1836), Bonnet (1837), and others determined the composition of pus by microscopic examination.

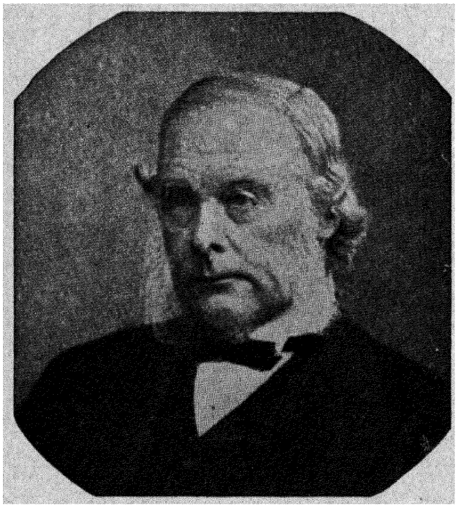

FIG. 252.—Joseph, Lord Lister. (*From Bulloch, "History of Bacteriology," Oxford University Press, London.*)

Coze and Feltz (1866–1869) injected putrid materials into dogs and rabbits and showed that blood from these animals, when injected into normal animals, produced similar symptoms. Their work showed that blood increased in virulence by animal passage. They succeeded in demonstrating the presence of bacteria in the blood of diseased animals and in persons suffering from typhoid fever, scarlet fever, puerperal fever, etc. Davaine (1872) confirmed the findings of Coze and Feltz.

The germ theory of disease was now gaining momentum, largely because of the work of Pasteur on the biological origin of fermentation. Lister greatly reduced the number of deaths from surgical infections by employing aseptic precautions, which was additional evidence in favor of the germ theory of disease. Davaine (1872–1879) injected a rabbit with a few drops of putrid blood. This resulted in death after 40 hr. He found that the transfer of blood from rabbit to rabbit for 25 transfers

increased the virulence enormously. Because of the progressive increase in virulence, the quantity of blood required to reproduce the disease in each succeeding rabbit became less. Final settlement of the relationship of microorganisms to disease was made by Koch (1878). He demonstrated, without a doubt, that diseases were due to microorganisms and that each disease was caused by a specific organism. Koch's success was due largely to the use of dyes for staining the bacteria and the condenser system invented by Abbé for increased illumination.

Ogston (1880) followed Koch's technique for the examination of over 100 acute and chronic abscesses for bacteria. Chronic abscesses gave negative results but acute abscesses showed the presence of groups and chains of organisms. Pus, heated or treated with phenol, was noninfectious but unheated or untreated pus gave positive results. He differentiated two kinds of cocci, namely, streptococci and staphylococci. Ogston's work was very complete and accurate and exerted a tremendous influence on the ideas of that period.

Henle (1840) believed that each disease was caused by a specific organism and that it could not be caused by any other organism. To show that an organism was the cause of a specific disease he postulated that the organism must be isolated and found capable of again reproducing the disease in another animal. These postulates are generally referred to as Koch's postulates because they were restated by Koch; in reality, they are Henle's postulates.

EARLY ATTEMPTS TO CLASSIFY BACTERIA

The first attempt to classify microscopic plants and animals was apparently undertaken by Müller (1773). He placed all of them in a group known as the "infusoria." Later classifications were proposed by Ehrenberg (1838), Dujardin (1841), Perty (1852), Cohn (1854), Zopf (1885), and Migula (1897). Cohn believed that bacteria showed more of the characteristics of plants than of animals and suggested that they be classified in the plant kingdom. Nägeli (1857) appeared to be the first to suggest the use of the term Schizomycetes (fission fungi) to include all organisms classified as bacteria.

Cohn (1872) believed that it was a difficult task to classify bacteria into genera and species. He pointed out that so many species looked alike that a classification based on morphology alone was insufficient. Physiological as well as morphological differences were necessary to distinguish bacteria accurately. This appears to be the first reference to the use of physiological characters in the classification of bacteria.

Cohn believed in the morphological fixity of bacterial species; otherwise he could not have classified microorganisms. Nägeli was not in accord with Cohn's views. He brought forth evidence to show that

every species of bacterial organism may appear in several types on the basis of morphological and physiological differences. Nägeli believed that one form could be converted into the other. Some of the scientists who were in harmony with the findings of Nägeli were Lankester (1873), Billroth (1874), Warming (1875), Zopf (1879), and Buchner (1882).

Zopf did not agree with Cohn's idea of the fixity of species; nevertheless he classified bacteria into four groups on the basis of morphology. The classification was as follows:

I. *Coccaceae*—spherical forms.

II. *Bacteriaceae*—spherical, rod, and filamentous forms, with no distinction between base and apex.

III. *Leptotricheae*—spherical, rod, and filamentous forms, the latter with distinction between base and apex.

IV. *Cladotricheae*—spherical, rod, and spirals, and showing dichotomy.

DISCOVERY OF THE ANTHRAX ORGANISM

Pollender (1849) and Davaine, working independently, first saw the anthrax organisms in the blood of cows affected with the disease. The rods were present in enormous numbers in the spleen. Brauell (1857) performed inoculation experiments and showed that the disease was transmissible from man to sheep and from horse to horse. Davaine, through a long series of researches, definitely proved that the rods were the cause of the disease anthrax. He found that the organisms were constantly present in the blood of animals suffering from the disease and that in the absence of the rods no illness was present. Since the germ theory of disease was not accepted by all scientists of that period, the work of Davaine did much to place it on a solid foundation.

DEVELOPMENT OF BACTERIOLOGICAL METHODS

Koch in 1876 took up the study of anthrax where Davaine had left off. He placed minute pieces of infected spleen tissue in drops of sterile blood serum and studied the cultures from hour to hour to see what took place. Koch saw the organisms grow in the form of long filaments inside of which appeared oval, granular bodies. He came to the conclusion that these bodies were spores. In order to rule out the possibility that the spores were contaminants he was able to show that they grew into anthrax vegetative cells. Koch showed that the disease was transmissible from mouse to mouse and that the lesions that appeared in the animals were identical.

Koch inoculated mice with organisms that were morphologically identical with the anthrax rods, but he was not able to produce the disease. He concluded that each disease was produced by a specific organism. Most of the early technical methods originated in his laboratory.

Staining Bacteria.—Various dyes for staining bacteria now began to be used. The first dyes were extracted from plants. The two best representatives of the group were carmine and hematoxylin or logwood. Coal-tar dyes first made their appearance about 1856. Hoffmann (1869) was probably the first to stain bacteria. He used carmine or fuchsin. Weigert (1871) employed méthyl violet for staining bacteria in histological sections. Salomonsen (1877) used fuchsin for staining bacteria.

The present-day methods for staining bacteria are essentially the same as those first advocated by Koch (1877). He prepared thin smears of bacteria on cover slips and dried them. The films were fixed in

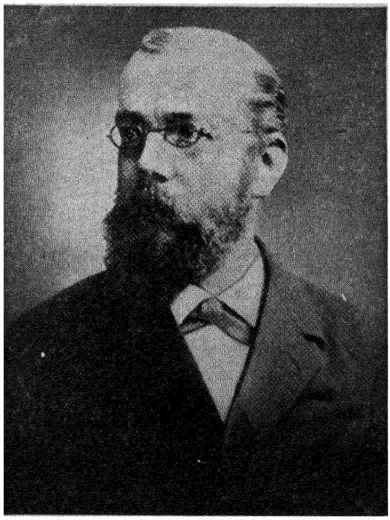

FIG. 253.—Robert Koch.

alcohol and then stained with various dye solutions such as methyl violet 5*B*, fuchsin, and aniline brown. He appears to have been the first to stain bacterial flagella.

Rapid progress on new staining procedures now followed. Weigert (1878) introduced Bismarck brown and Ehrlich (1881) methylene blue. Koch employed methylene blue when he discovered the tubercle bacillus in 1882. Loeffler (1884) added a small amount of potassium hydroxide to a solution of methylene blue to neutralize the acidity of the preparation. This solution is known as Loeffler's methylene blue and is extensively employed at the present time. Since the methylene blue now prepared in this country is neutral in reaction, the addition of the alkali is no longer necessary. Potassium hydroxide may cause too rapid oxidation of the methylene blue, resulting in a solution with poor keeping

qualities. In 1882 Ehrlich first announced the method for staining the tubercle bacillus. He added aniline oil to methyl violet or fuchsin as an intensifier. Smears stained with these solutions revealed the presence of long, slender, deeply stained, rod-shaped organisms. Ehrlich found that the organisms were not decolorized when treated with 30 per cent nitric acid so he called them "acid-fast." He used vesuvin as a counterstain for methyl violet, and methylene blue as a counterstain for fuchsin. Ziehl substituted phenol (carbolic acid) for the aniline oil and Neelsen decolorized with sulfuric acid instead of nitric acid. This modification is known as the Ziehl-Neelsen staining solution. In 1884, Gram discovered the staining technique bearing his name. He was working on a new method for staining bacteria in tissues, but discovered a new differential stain for bacteria.

Cultivation of Bacteria.—The development of various staining procedures was followed by studies on the nutritive requirements of bacteria. Pasteur employed carbohydrate media. Miquel (1883) suggested the use of Liebig's beef extract or an infusion prepared from lean beef. Loeffler (1881) cultivated organisms on a medium composed of meat infusion to which was added 1 per cent peptone and 0.6 per cent sodium chloride. The reaction was made weakly alkaline with sodium phosphate buffer. This is essentially the composition of nutrient broth used at the present time. Koch (1881) worked on the perfection of solid media, which were required for use in his isolation experiments. He finally recommended the addition of 2.5 to 5 per cent gelatin to nutritive fluid media. Two disadvantages to the use of gelatin are that it is fluid at body temperature and that it is liquefied by many microorganisms. At the suggestion of Frau Hesse, the wife of one of Koch's assistants, agar was substituted for the gelatin. This overcame all the objections of gelatin as a solidifying agent. In 1882 Koch introduced coagulated blood serum as a solid nutritive medium for the cultivation of the tubercle bacillus. Koch poured melted solid media on glass plates and placed them under bell jars to prevent aerial contamination. Petri (1887) recommended the use of covered dishes bearing his name. Petri dishes or plates have not been altered since the time of Koch and are standard equipment in bacteriological laboratories at the present time.

Isolation of Species.—The separation of organisms into pure species was first attempted by Klebs (1873). He introduced a small amount of inoculum into a sterile medium. When visible signs of growth appeared, a small amount of the culture was transferred to fresh medium. This was repeated several times. He believed that one organism would eventually overgrow the others with the result that a pure culture would be obtained. Hoffmann (1865) employed solid media in the form of pieces of potato and bread. Schroeter (1872) used such solid substances

as potato, starch paste, flour, egg albumin, and meat. Koch also employed potato medium but greatly improved its preparation. Brefeld (1875), working on pure cultures of molds, laid down the rules required to separate them into species: (1) The inoculation of a sterile medium should be made from a single spore. (2) The medium should be favorable to the growth of the species. (3) The culture should be protected from aerial contamination. The method was difficult to apply to the isolation of pure bacterial species because the cells are considerably smaller than mold spores. Lister (1878) made high dilutions of a culture in sterile medium in the hope that a growth from a single organism would eventually be obtained. His method proved successful and was adopted by Hansen (1883) for the propagation of pure species of yeast.

Coze and Feltz (1866), Davaine (1872), and Koch (1876) employed the method of animal inoculation for the isolation of pathogenic organisms. Koch showed that the anthrax organism could be separated from other nonpathogenic species by injecting the mixed culture into mice. Only the anthrax organism invaded the tissues of the host, with the result that a pure culture of the organism could be recovered from the blood of the diseased animal.

Koch devoted considerable time to a solution of the problem that was to place him in the front rank as a bacteriologist. He first used various nutritive media solidified by the addition of gelatin. Koch poured melted nutrient gelatin on sterile glass plates and placed them under bell jars until firm. He inoculated a sterilized needle with some of the mixed culture and streaked it over the surface of the solidified gelatin. Different types of bacterial colonies soon appeared on the plates, each colony arising from a single organism. This is known as the streak plate method. Later Koch (1883) mixed some of the culture with the melted gelatin and then poured it on sterile glass plates. In this procedure the colonies were embedded in the gelatin instead of being present only on the surface. The separation was much better by this procedure. This is known as the pour-plate method. The methods Koch advocated are the same as those used today, except that agar is now universally employed in preference to the gelatin for reasons already given.

Sterilization by Filtration.—Tiegel (1871) appears to be the first to use filtration as a method for the removal of bacteria from liquids. He passed anthrax cultures through a porous cell composed of unburned clay. Pasteur and Joubert (1877) substituted plaster of Paris for the clay. Miquel and Benoist (1881) used a mixture of asbestos and plaster of Paris. Chamberland (1884) employed cylinders composed of unglazed porcelain. This type of filter is still widely used under the name "filtre Chamberland, système Pasteur." In 1891 Nordtmeyer made a filter

of infusorial earth or kieselguhr. These are named Berkefeld filters after the owner of the mine from which the material was first obtained.

ATTENUATION OF BACTERIA

The pioneer discoveries in this field were made by Pasteur. He isolated the organism of chicken cholera and cultivated it in pure culture. Injection of the organisms into normal chickens usually resulted in a fatal infection. Occasionally chickens recovered from the disease and became refractory to a second inoculation. The first dose conferred sufficient immunity to protect the chickens from a subsequent infection. Pasteur noted that the virulence of a culture depended to a large extent upon its age. As the time between transfers was increased the ability of the organisms to produce an infection became less. If he used very old cultures, the organisms failed to produce cholera in chickens but still retained the ability to immunize the fowls against the disease. He used the term "attenuated" to describe such cultures. This was probably the first experiment performed to demonstrate the value of an attenuated culture for the elaboration of immune bodies in the host without the appearance of symptoms of the disease. This same principle was applied to the preparation of vaccines of the organisms of anthrax, swine erysipelas, and rabies.

Pasteur devoted his attention next to a study of anthrax. He found that the organism did not grow at a temperature above 45°C. but did grow at 42 to 43°C. A virulent anthrax culture kept between 42 to 43°C. failed to produce disease in laboratory animals but was still capable of eliciting an antibody response. This attenuated culture of the anthrax organism was used as a prophylactic, to protect animals from a subsequent dose of virulent bacilli.

His next problem was concerned with the disease known as swine erysipelas. A well-defined bacillus was discovered in the blood of pigs suffering from the disease. Attenuation was accomplished by passage of the organisms through the bodies of rabbits. Inoculation of pigs with this attenuated vaccine did not produce the disease, but did confer protection against a subsequent dose of virulent organisms.

Perhaps the crowning achievement of Pasteur's work was his investigations on rabies. It was shown that the virus of the disease attacked the central nervous system. The incubation period of the disease varies from 40 to 60 days. Pasteur inoculated the virus into dogs directly under the dura mater and showed that the incubation period before symptoms appeared was greatly shortened. He noted that when dogs had recovered from the disease they became immune to a second inoculation. Pasteur prepared his vaccine by removing the spinal cords of rabbits dead of the disease and by drying them for varying periods of time.

As the drying period was increased, the virulence of the virus decreased. In this manner he prepared virus of varying degrees of virulence. The virus employed for the first injection was dried for 14 days. Pieces of the cord were emulsified and injected into a patient bitten by a rabid dog. For 12 more daily injections virus of gradually increasing virulence was employed. Finally, pieces of cord, taken from an animal dead of virulent virus, were emulsified and injected. In this manner immunity was conferred upon the patient and symptoms of the disease did not develop. If treatment was not begun early, the immunity conferred by the vaccine was not always sufficient to destroy the virus before it reached the central nervous system with the result that symptoms of the disease appeared. This treatment, with slight modifications, is now used in all parts of the world and has reduced the incidence of rabies to a very small figure.

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